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„Development and implementation of a shRNA screen for inhibitors of the pRB and p53 pathways in mammalian cell cultures“

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1 Zusammenfassung

Rund 50% der von Menschen abstammende Tumoren besitzen eine mutierte Form des TP53 Gens das für den TP53 Tumorsupressor kodiert[1]. Seit der Entdeckung dieses "Wächter des Genoms" suchen sowohl pharmazeutische als auch akademische Laboratorien nach gezielte Therapien um Tumoren mit einer defekten Kopie des P53 zu beseitigen[2]. So viel versprechend dieser Strategie sein mag, bleiben der restlichen 50% der Tumoren die einen Wildtyp TP53 besitzen davon unbetroffen.

Viele der in den p53 Apoptosesignalweg verwickelte Gene wurden durch funktionelle zelluläre genetische Screens gefunden[3, 4, 5]. Die Meisten dieser Screens basieren auf einen ähnlichen Prinzip, nämlich durch Hemmung eines p53 aktivierenden Genes, das p53 Signalweges auszuschalten und Zellen in besitzt eines Kandidatengenes aufgrund ihrer "Unsterblichkeit" zu isolieren. Auch wenn die gewonnen Einblicke in der apoptotische Signalweg viele neue Erkenntnisse gebracht haben, hielt sich die klinische Relevanz von den gefundenen Gene in Frage. Viel sinnvoller dagegen ist es solche Gene zu finden die bei Inhibierung, eine p53 aktivierende Wirkung zeigen um dadurch Zellen dazu zwingen in Apoptose zu gehen oder den Zellzyklus zu arretieren. Dies wurde bereits bereits bei den chemischen Hemmung des HDM2 Gens durch Nutlin-3 gezeigt[6, 7].

Während meiner 12-monatige Diplomarbeit, begab ich mich einen "Loss-of-Function" genetischen Screen zu entwickeln um weitere Inhibitoren der p53-Signalweges zu finden mit der Idee, neue Angriffspunkte für Medikamente für die Krebstherapie zu identifizieren. Meinen Auftrag war ein Selektionssystem zu testen wodurch Zelllzyklus arretierte Zellen gegenüber sich teilende Zellen spezifisch ausgesucht werden sollten. Der Screen besteht in Grunde aus vier spezifische Schritte: Als erstes wird einer p53 Wildtyp, immortalisierte Zelllinie mit einer shRNA Bibliothek bestehend aus 24.000 retrovirale Vektoren gegen 9.000 menschliche Gene infiziert. Die Zellen werden dann im zweiten Schritt einer Behandlung unterzogen wodurch sich Teilende spezifisch getötet werden. Dies wird erzielt durch Zugabe vom synthetischen nukleosid Bromodeoxyuridin (BrdU), dass während der S-Phase des Zellzyklus von der Polymerase in den Genom statt Thymidin inkorporiert wird. Die Zellen werden anschliessend mit der Hoechst #33158 Farbstoff behandelt und nach einer kurze Inkubationszeit mit UVa licht bestrahlt und dadurch werden DNS Doppelstrandbrüche verursacht[8, 9, 10]. Zellen dessen Zellzyklus arretiert wurde durch die Wirkung eines shRNAs werden von der Doppelstrandbrüche verschont, die Selektion dieser Zellen wird im dritten "Auferstehungsschritt" ermöglicht. Nun werden überlebende Zellen mit einem lentiviral exprimierten Large T Antigens infiziert mit der Erwartung die Effekte des shRNAs zu übertrumpfen und die Zellen somit zu stimulieren wieder in Zyklus zu gehen und kolonien bilden. Genomisches DNA wird aus den Kolonien isoliert und der shRNA vektor kann durch PCR identifiziert werden.

2 Aim

In humans, roughly 50% of tumours possess a mutated form of the TP53 gene which encodes the TP53 tumour suppressor[1]. Thus, since the discovery of this "Guardian of the Genome", both pharmaceutical and academic research laboratories have been thoroughly looking for ways to target tumours with defective P53[2]. However, there is still the other 50% of tumours which have a wild type TP53.

Various functional genetic screens have identified genes that suppress p53 mediated cell cycle arrest or apoptosis[3, 4, 5]. Although important for our understanding of the mechanisms of action of these pathways, the therapeutic value of these findings is limited. Instead it would be preferable to find genes that activate p53 when inhibited. As has been shown with the small molecule Nutlin-3, inactivation HDM2, an upstream negative regulator of p53, can activate the p53 pathway and induce cell cycle arrest and apoptosis in cellular models[6, 7]. During the 12 month period of my diploma thesis, I set out to develop a loss-of-function screen to identify further inhibitors of the p53 molecule with the idea of finding novel drug targets for cancer therapy.

In the past, these screens have been hampered by the inability to recover knock down vectors from arrested cells. Thus, we propose a strategy to circumvent this problem and will perform a genome-wide shRNA screen to identify inhibitors of the pRB/P53 signalling network. We will infect immortalised cells with the retroviral shRNA NKi library (9,000 genes targeted) at a low MOI and then submit the cells to a treatment that will kill all dividing cells. This is accomplished with the help of Bromodeoxyuridine (BrdU), a synthetic nucleoside which is incorporated into the genome of a replicating cell in substitution of a thymidine. Several studies have shown that double strand breaks can be induced in BrdU labelled cells by a subsequent treatment with Hoechst dye #33158 and UVa light[8, 9, 10]. We postulate that the BrdU/Hoechst/UVa induced double strand breaks will kill or arrest all dividing cells, whereas non-dividing cells which are arrested by one of the shRNAs from the NKi library should not be affected. All surviving arrested cells will then be infected with a lentiviral vector containing the SV40 Large T antigen under the control of a CMV promoter. Colonies are expected to form where the arrest has been induced by a shRNA that targets an inhibitor of the p53/Rb pathway, since both p53 and Rb proteins are inhibited by the large T protein. Genomic DNA from colonies will then be isolated and the knockdown vector recovered by PCR. If the shRNA recovered is proven to by relevant and novel, we will further study its function and role in the pathway in cooperation with other labs of CeMM.

3 Introduction

3.1 Cancer

The most read and trusted reference place on earth, Wikipedia.org defines cancerous as opposed to benign tumours as being a "class of diseases in which a group of cells display *uncontrolled growth, invasion,* and sometimes *metastasis.*" As simple as it is, this definition accurately summarises the three key events in cancer progression, effective targeting of a cancer at any of these events will thus be correlated with a good disease prognosis. All three events are equally important and subject to extensive research, the focus of my project was on finding a mechanism to halt the uncontrolled growth of cancer cells.

Pathologically, cancer incidence correlates with age as our genomes and proteomes are more prone to malignant aberrations the older we get. The International Agency for Research on Cancer estimated that for the year 2008 there were worldwide 7.6 million deaths from cancer[11]. Due to our ever improving living standards, it is very plausible that our life expectancies as a species will increase in the foreseeable future[12]. To put this in context, until the relatively recent discovery of antibiotics, millions of years of evolution had fine tuned the human species to a life expectancy of a maximum of 40 years of age. Since the beginning of the 20th century there has been a huge rise in age expectancy (Figure 1a). This trend could also be correlated with the increasing number of cancer deaths worldwide (Figure 1b).

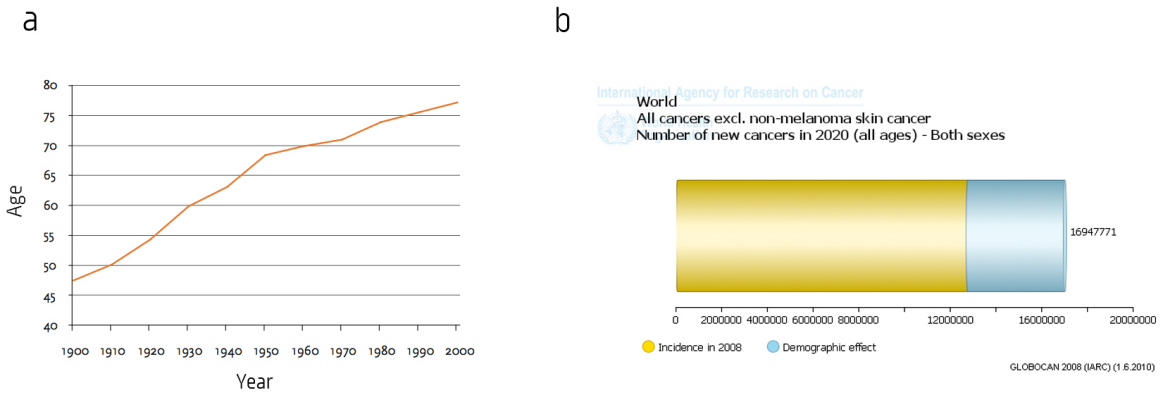

Figure 1: *Correlation of age expectancy and cancer incidence.* **a)** Since the beginning of the 20th century there has been an exponential growth in the life expectancy, in part due to improving hygienic standards and by the discovery of antibiotics. Adapted from [13].**b)** Projections from the IARC estimate that four million more cancer cases will be diagnosed in 2020 as compared to 2008.

Biologically, cancer can be best understood if seen as the "escape" of a cell or group of cells from its intrinsic function in the higher complexity of an organism to an individual entity. This new entity possesses the qualities of an aggressive parasitic organism, in that it will try its best to survive in some cases by diverting valuable energy resources from the hosts body to feed its own needs, even if this eventually leads to the death of its host. Inevitably a selective pressure is impinged upon both the cancer and the host and both naturally evolve mechanisms that ensure survival. Thus every cell in a multicellular organism has over the course of time evolved a wide variety of intrinsic safeguards that protect the organism as a whole from such cancerous cells. However, as a replicating entity, individual cells also possess a drive to reproduce. What from the multicellular organisms' point of view may seem as a failure might be a victory for the individual cell.

To understand the series of events leading to the uncontrolled growth of a cell it is essential to first elucidate the intricate molecular pathways governing the progression of the cells' most ancient mechanism: the ability to reproduce. The key steps in this process are governed by the cell cycle.

3.2 The cell cycle

Every single cell in the body is originally derived from a single cell generated during fertilisation. The series of events that lead to cellular division is defined as the cell cycle. Figure 2 is a schematic and simplified depiction of the cell cycle with its respective checkpoints. Each new event in the cell cycle can only begin once the checkpoints of the preceding steps have been cleared. At the center of most if not all cancers lies a mis-regulation in the cell cycle.

Normally, cells in the body will divide and differentiate until the final state of the given cell lineage is reached, after which the cell will be purposely stalled in one of the cell cycle steps. This arrest is crucial in order to keep the homeostasis of cells in the body, however it is also an essential defence mechanism against cells attaining cancer-inducing mutations. The genetic information within the cell remains inactive, thus it is within reason to theorise that even after full differentiation every single cell retains the potential ability to divide mitotically. Indeed, the recent work of Yamanaka has shown that a pluripotent state can be artificially induced in fully differentiated fibroblast cells [15, 16].

A myriad of mechanisms are in place that meticulously regulate cell cycle progression, and there are two seemingly central proteins on which many of the checkpoint signals impinge: P53 and Rb.

3.2.1 p53

The TP53 gene itself is mutated in roughly 50% of tumours which make it the most frequently inactivated protein in human tumours analysed. Furthermore, an ever growing number of post-translational miss-regulations which inactivate the wild-type p53 gene product, leave no doubt that the p53 protein is worthy of its "Guardian of the Genome" nickname[17]. It is specifically the post-translational regulation of the p53 pathway, which has recently gained the attention of the scientific community after several high profile studies showed that the activation of p53 in tumours can be readily achieved by inhibition of p53 inhibitors. The central inhibitor of the p53 protein is the MDM2 protein[18]. MDM2 binds to p53 and inhibits its function by (1) inhibiting the transactivation domain of the p53 protein, (2)promoting the proteasomal degradation of p53 through its E3 ubiquitin ligase function, and (3)through nuclear export of p53 (Figure diagram of p53-MDM2 interactions). Any form of p53 inhibition has the potential to promote cell cycle progression making MDM2 a very potent oncogene. Interestingly Yamanaka's group recently showed that a reduction in the p53 levels increases the efficiency of IPS cell generation both in human and in mouse cells[19].

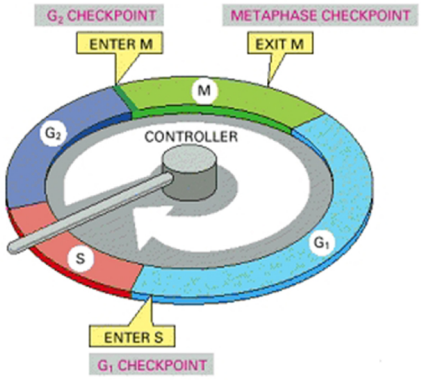

Figure 2: *Schematic depiction of the cell cycle.* Each phase of the cell cycle is rigorously controlled for completion. When an aberrant event is detected the cell cycle will stall until the errors are corrected or until a bypass signal is received. Modified from Alberts Molecular Biology of the Cell[14].

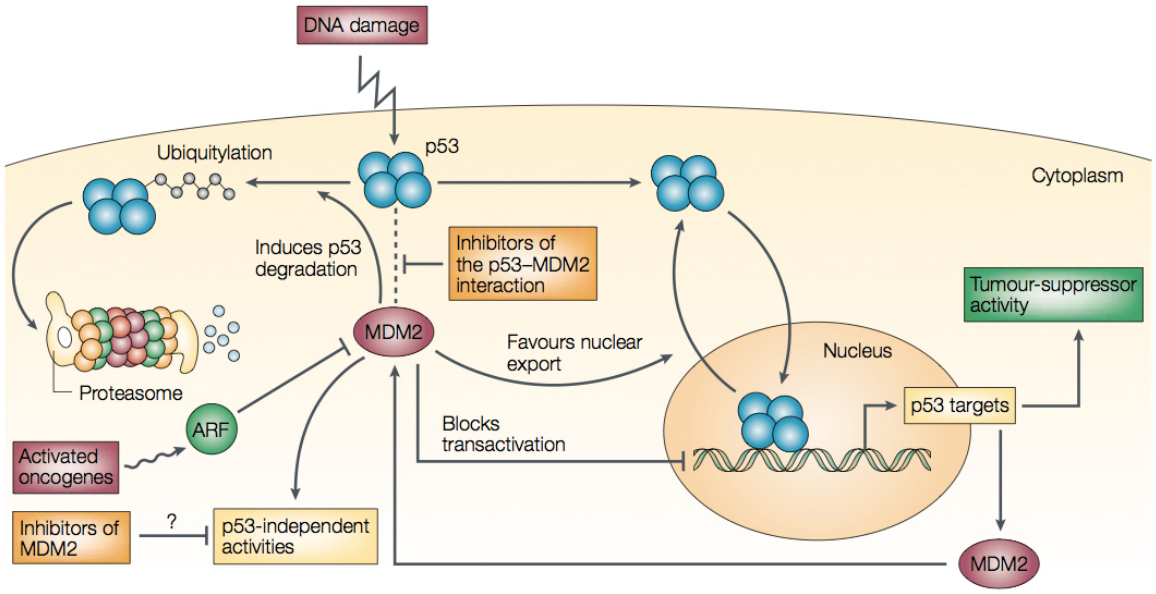

Figure 3: *Regulation of p53 activity by MDM2.* The MDM2 protein plays a central role in the regulation of the p53 tumour suppressor.Adapted from Chene 2003[2].

3.2.2 RB

The second central protein in the regulation of cell cycle progression, is the retinoblastoma (RB) protein. Important downstream effectors of RB are the E2F family of genes. These genes were initially associated with the regulation of the G1/S-phase transition, more recent studies have implicated these genes in apoptosis, differentiation and DNA-Damage signalling pathways[20]. RB possesses at least 16 CDK phosphorylation sites, and its activation status depends on its phosphorylation state [21]. As the cell cycle progresses RB is phosphorylated by members of the cyclin dependant kinases family (CDKs), the hypophosphorylated state of RB is restored by pyrophosphatase 1 (PP1) after DNA replication has been completed. RB belongs to a family of structurally and functionally related proteins which include p107 and p130, which functions however are different depending on the cellular context [22]. A striking difference for example is that the RB gene, in contrast to p107 and p130, is frequently mutated in cancerous tissue, and loss of RB function has been shown to be involved from cancer initiation to cancer progression[23]. As with p53, post-translational modifications of the RB gene add to malignant miss-regulations in the pRb pathway. For example, over-expression of Cyclin-D or CDK4 kinases will lead to enhanced Rb phosphorylation and thus cell cycle progression[24].

3.2.3 Links between p53 and Rb

Although structurally unrelated, the overlap between p53 and RB functions impossibly escapes attention. The regulation of these two pathways is extensive and diverse but some intriguing convergences of the pathways do occur. I will shortly mention two of these examples in the following paragraphs.

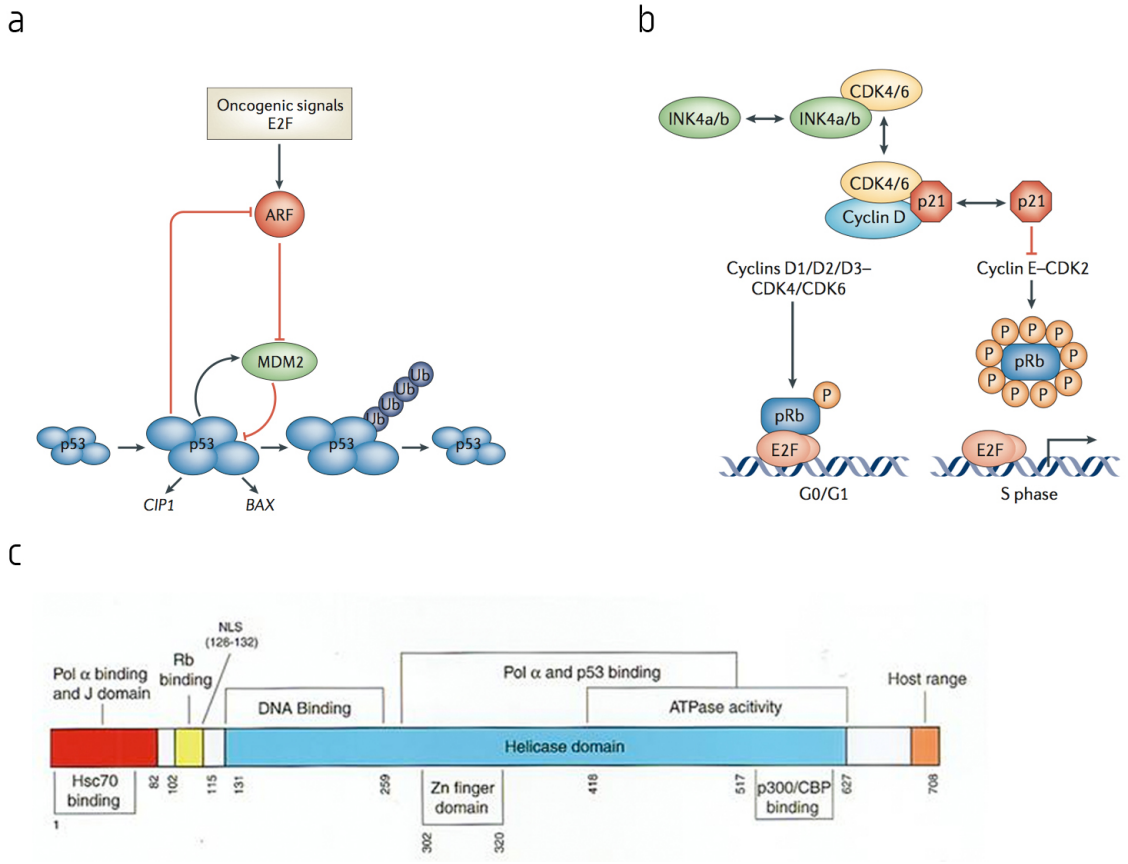

Figure 4: *Links between p53 and Rb pathways.* **a)** Oncogenic signals can induce the ARF tumour suppressor which in turns inhibits MDM2 thus hindering the inhibition of p53.**b)** Similarly, the INK4a/b tumour suppressor inhibits the CDK4-Cyclin D which in turn cannot inactivate p21 hindering the hyperphosphorylation of RB. is not complex. Adapted from [25].**c)** Schematic depiction of the Large T antigen. Adapted from [26].

3.2.3.1 INK4a ARF locus First, an important regulator of these two pathways is the $p16^{INK4a}/p14^{ARF}$ locus which encodes for three critical tumour suppressors INK4a INK4b and ARF. The two INK4 sequences are arranged in tandem, but are separated by an exon designated 1β which is transcribed incorporating exons 2 and 3 from $p16^{INK4a}$, albeit in a different reading frame thus the name Alternative Reading Frame (ARF) protein. ARF has no amino acid sequence or biochemical function homology to $p16^{INK4a}$ and the evolution of the gene locus remains a mystery. The gene products of $p16/15^{INK4a/b}$

work by inhibiting CDK4 and CDK6 which thus cannot phosphorylate Rb and the cell cycle is arrested in the G_0/G_1 phase (Figure 4a), the ARF protein binds to MDM2 inhibiting its ubiquitin ligase activity thus stabilising the p53 protein (Figure 4b)[25, 27]. One would expect the expression patterns of the $p16^{INK4a}$ and $p14^{ARF}$ gene products to be similar, however extensive studies have not been able to show this in any cellular model [25].

3.2.3.2 SV40 LargeT antigen The second example of convergence between the p53 and Rb pathways, is an extrinsic rather than an intrinsic one. Viruses have been perfecting the art of tinkering with the cellular replication machinery for millions of years. Many DNA tumour viruses have developed special viral proteins to bind and inactivate Rb or p53 such as the E7/E6 proteins from the human papiloma virus (HPV) family or the Large T antigen from the simian vacuolating virus family (SV40). In the case of the SV40 virus, its large T (LT)-Antigen protein is a single multifaceted protein which binds and inhibits both p53 and Rb thus stimulating early S-Phase entry and its own replication. In addition, LT interacts with a variety of other cell cycle related proteins, like MDM2, p300/CBP, CUL7, BUB1, TEF-1, NBS1, FBW7, as well as HSC70(Figure 4c)[28].

3.2.4 The therapeutic potential of targeting p53 or RB post-translational inactivators

The focus of several recent high impact publications has been the activation of either the Rb or the p53 pathways to activate the cells natural cancer defence mechanism. These studies have focused on finding inhibitors of p53/Rb inhibitors. Surprisingly, although activation of these pathways in cancerous cells leads to cell death and apoptosis, no adverse effects have been shown in normal cells.

For the p53 protein, scientists have succeeded in creating small chemical inhibitors which disrupt the MDM2-p53 interaction[6, 29, 30]. The most promising chemical inhibitor candidates are Nutlin3a and M-219. P53 wild type cancer cells treated with any of these two compound show up-regulation of p53 and its putative target p21, and induce p53 dependent cell death by apoptosis. Both compounds show achieve this with a very high affinity.

As of today no such central inhibitor for Rb has been found, as HDM2 is for p53. Investigation into disrupting the interaction of several of the cell cycle kinases has yielded only moderate results thus far[31]. The Raf-1 kinase was found to bind and phosphorylate

Rb very early in the cell cycle, promoting its activation [32]. Davis and colleagues correctly hypothesised, that a small chemical inhibitor disrupting the formation of the Raf-1 Rb interaction would inhibit an activated Rb thus prompting cells to go either into cell cycle arrest or apoptosis. Indeed, cells treated with the specific Raf-1 inhibitor RRD-251 showed precisely this phenotype and nude mice bearing human cancer xenografts showed a retraction of the tumour at physiologically relevant concentrations[33, 34].

3.3 Functional loss-of-function genetic screens in mammalian cells

Functional genetic screens in model organisms such as yeast, fruit-fly and worms have provided a wealth of information about components of the mammalian signalling system[35]. However, there are limitations to translating the results of these screens to human beings. The use of mammalian cells to study cancer development had until recently been hampered by the lack of effective genetic tools. However, the number of tools that have been developed has increased dramatically in the last years. The fastest growing of which without a doubt has been the discovery and further implementation of small interfering RNAs.

3.3.1 RNAi knockdown libraries

Loss of function genetic screens aim to identify gene functions through inactivation of a gene or its corresponding mRNA. Various technologies exist to suppress genes in mammalian cells among which RNA interference has proven to be the most effective and reproducible. RNAi is an ancient defence mechanism to protect cells from foreign invasion agents such as transposons and viruses. Double-stranded RNAs produced by these agents are processed into short double-stranded RNAs by DICER, which in turn signal the RNA-induced silencing complex (RISC) which in turn degrades homologous mRNA molecules[36]. By making artificial sequences targeting an ORF of a target gene, scientists have been able to pointedly silence an ever growing number of genes[35] . However, this same mechanism makes the use of RNAi in mammalian cells a problematic issue since the introduction of long double-stranded RNA molecules leads to nonspecific toxicity mediated by the γ-interferon pathway[37].

Generally, two ways have been developed to overcome this toxicity. First, the transient transfection of 21-23 base pair double stranded siRNAs which directly recruit the mammalian RISC without eliciting γ-interferon response [38]. Some hurdles however are the transient nature of these constructs, the difficulty to transfect i.e. primary cells,

and the high cost of synthesising siRNAs. Vector systems that mediate stable production of siRNA-like molecules in mammalian cells overcome the problems of siRNA transfections[39]. Most of these vectors use RNA-polymerase III promoters to direct the synthesis of short hairpin RNA (shRNA), consisting of a double-stranded stem of 19-29 base pairs targeting the mRNA to be suppressed connected by a loop of 6-9 bases. These molecules are processed intracellularly into siRNA-like molecules. Stable integration of these expression cassettes into the host genome can be achieved through retroviral or lentiviral delivery vectors[40].

3.3.1.1 The NKI RNAi knockdown Library The RNAi library used in my project, was produced through high-throughput cloning of short-hairpin-encoding DNA oligonucleotides into a retroviral vector (pRetroSuper) with the RNA-polymerase III promoter at the Netherlands Cancer Institute (NKI for its abbreviation in Dutch)[5]. It consists of 23,742 knockdown vectors targeting 7,914 human genes. Each mRNA transcript is targeted by three sequence independent vectors, thus increasing the likelihood of functional inhibition of gene expression. A further important characteristic of shRNA libraries is the ability to isolate and identify the individual hairpins after genome integration and siRNA expression. Accordingly, all the 19-meres in the NKi RNAi library are flanked by common primer sites within the LTRs. These allow for an easy recovery through conventional genomic DNA PCR, the 19-mer itself then serves as a unique barcode identifier.

3.3.2 Previous screens: Finding genes that induce senescence

Previous screens have focused on finding candidate genetic interactions that inactivate the p53/Rb proteins thus bringing cells back into cycle. One of the very first loss of function screens in mammalian cells was performed by the group of Rene Bernards and is outlined in Figure 5 [5]. For their approach they generated a hTERT immortalised primary human foreskin fibroblast cell line containing a temperature sensitive allele of the SV40-large T antigen (from know on BJ-TERT-tsLT cells). Culturing the cells at 32°C the active Large T permits cells to grow, switching the temperature to 39°C causes synchronous p53 dependent cell cycle arrest and apoptosis. The Bernards' group infected the BJ-TERT-tsLT cells with the NKi library and screened for cells that would grow at 39°C implying the escape of senescence in a p53 Rb dependent manner.

Similar to the approach by Bernards, a second screen performed by the group of David Bernard, focused on shRNAs that would allow candidate cells to escape the senescent state [3]. In their setup, they infected human lung fibroblast cells (WI38) known to

undergo senescence after 50 ± 10 cycles, with a shRNA library and screened for cells that escaped senescence.

Although these screens provide insight into key pathways involved in the cell cycle, the resulting information is of limited therapeutic value. As mentioned before, the desirable effect to elicit in cancer biology is to stop the erratic cell divisions by either arresting the cells at some point in the cell cycle or by specifically killing the cells. In order for the candidate genes identified by both the Bernards and the Bernard groups to elicit such a response, the genes or gene products must be activated which is not a trivial matter.

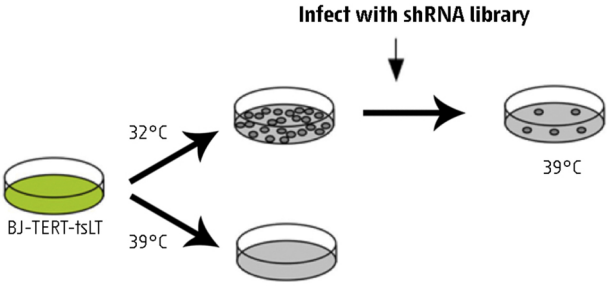

Figure 5: *Outline of mammalian RNAi screen to identify inducers of cell cycle arrest.* BJ-TERT-tsLT cells will grow at 32°C and arrest at 39°C. By infecting actively dividing cells with the shRNA and then switching to the non-permissive temperature, some cells will escape the arrest and grow into clonal colonies. The knockdown eliciting this phenotype can then be easily recovered through PCR. Modified from [5]

3.3.3 Our approach: Finding Genes that evade senescence

As of today most drugs are designed to disrupt the function of a gene or gene product. Thus, of a much higher therapeutic value would be to identify genes that confer a cell the ability to bypass the inherent cellular safeguard mechanisms, and to inhibit the gene's function. The identification of such a gene, if proven to be involved in a central regulatory pathway, could eventually lead to a specific drug target. On this account, my master thesis project focused on developing a screening method that would single out genes that elicit a senescent phenotype in cells.

The novelty of our approach lies with the ability to screen for genetic interactions that activate the cells intrinsic defence mechanism by activating either p53 or Rb. I attempted to accomplish this by developing a **four-step** screening technology (Fig. 6). The four steps of our approach are:

1) The **knockdown** of genes with a shRNA library.
2) The systematic **killing** of all cells which are still dividing.
3) Stimulating cells which survived the previous step to **re-cycle**.
4) **Identifying and confirming** the shRNA responsible.

3.3.3.1 Knockdown The first step consists of knocking down a wide array of genes by infecting the desired cells with the NKi shRNA knockdown library described in 3.3.1.1. We hypothesise that a senescent phenotype will be induced by some of the roughly 8,000 genes silenced by the library.

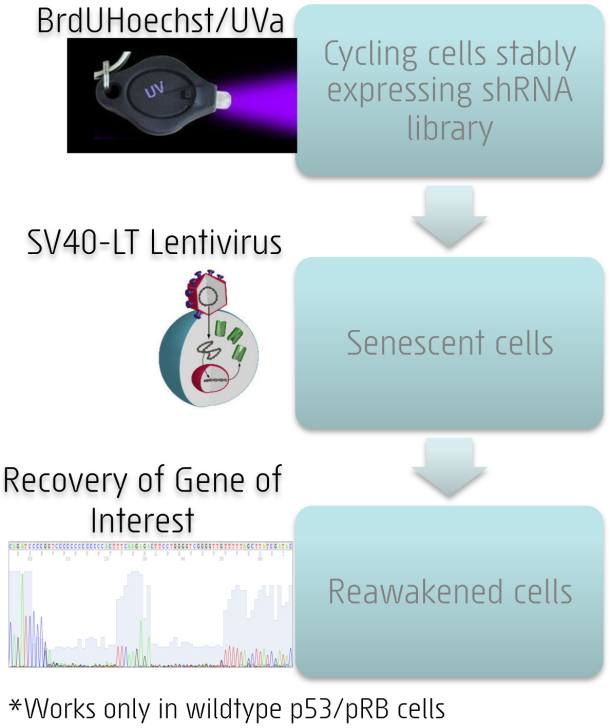

Figure 6: *Schematic diagram of the four-step approach.* Cells infected with the NKi retroviral library are submitted to the BrdU/hoechst/UVa treatment which will kill all dividing cells. Remaining cells are then infected with a lentiviral vector expressing the SV40 Large T antigen under the CMV constitutive promoter. Cells which are rescued from senescence by the Large T antigen will divide until colonies are formed. The genomic DNA of the individual colonies is analysed and the hairpin sequence is recovered by PCR.

3.3.3.2 Killing After giving an appropriate time for the infected cells to properly express the introduced shRNA and acquire the desired senescent phenotype, the cells will be subjected to a treatment that selectively kills cells that are still undergoing the full cell cycle. We attempt to do this by implementing a previously described method to induce double strand DNA breaks in cells treated with a combination of 5-bromo-2'-deoxyurine (BrdU) the Hoechst 33 258 dye and Ultraviolet A light. The exact protocol can be found in the *Materials and Methods* section and in: [41, 8, 42, 10] . The method is based upon the finding by Limoli and collegues, that cells which have incorporated the thymidine analogue BrdU into their genomes during S-phase were more sensitive to UVa radiation than cells which had not [42]. Further treatment of these cells with the Hoechst 33258 dye enhances the effect to the extent where almost 100% of cells with incorporated BrdU are selectively killed. We hypothesised that if we could accomplish a similar efficacy in killing dividing cells, only cells arrested by an shRNA from the previous step would survive.

3.3.3.3 Re-cycle As was demonstrated by the BJ-hTERT-LTts cell line, cells can be forced into cycle in a p53 dependent manner with the help of the SV40 Large T antigen[5]. Thus we hypothesised that by introducing an active form of the Large T antigen into cells which have been arrested, we could overcome the arrest and once again the cells

would start dividing. Our hypothesise is however based on two presumptions, first of all, that the cells have gone into cell cycle arrest due to the silencing of an essential gene in the p53 or Rb pathways. Secondly, the effect of the Large T mediated inactivation of p53 or Rb must be dominant to the activation of the pathway by the hairpin. In cells where both premises are fulfilled, the cell cycle will be kick started again and individual clonal colonies should grow out.

3.3.3.4 Identify and confirm The clonal colonies are cultured until a sufficient cellular mass is achieved, after which genomic DNA (gDNA) is isolated and using the universal primer pairs common to every hairpin and the gDNA as a template, a PCR is performed. The product of the PCR is then sequence analysed using the same primers as for the amplification, with the help of the basic local alignment search tool (BLAST) the 19-mer sequence is assigned to its target mRNA. After a successful identification, it is crucial to confirm whether the senescent phenotype was indeed induced by the hairpin. The confirmation step is explained in detail in the *Results* section.

4 Results

4.1 Setting up the screen

As explained in the previous sections, the screening method consists of a four-step procedure. Although the technologies we intended to implement for our screen had been previously published, this by no means implies that they will all work under every experimenters hands or in all cell systems. Thus, the first months of my thesis were dedicated to finding the optimal conditions for each of the four steps described in section 3.3.3. The highest priority when performing a large scale study is to carefully choose which model organism best suits the problem at hand. Our screening methods were conceived for use in cell lines kept in culture. There are an enormous variety of available cell lines for research purposes, thus as a first step it was essential to find the appropriate cell system to study.

4.1.1 Cell lines

Independently of the original cell line's source, it is absolutely necessary that the p53/Rb proteins be wild-type for the Large T to be able to act. Furthermore, BrdU/Hoechst/UVa treatment should efficiently kill more than 99% of cycling cells. In order to not spend more time than needed in establishing the proper handling of a newly acquired cell line, we decided to search among the available cell lines in our lab for an appropriate candidate. The p53 Rb wild-type requirement on itself narrowed our search down to three candidate cell lines: U2OS, MCF10a and IMR90 which are individually described in the *Materials and Methods* section and depicted on Figure 7.

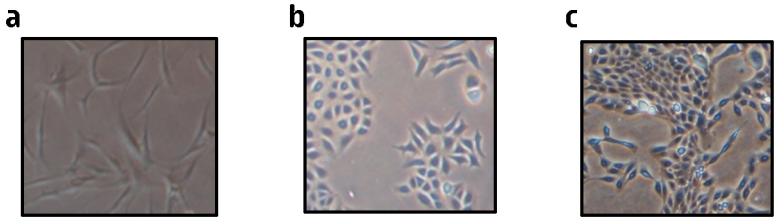

Figure 7: *Cell lines proposed to perform the screen.* Phase contrast microscopy photographs at 40x magnification of a human embryonic fibroblast IMR90 **a)**, a human osteosarcoma cell line U2OS **b)** and a non tumourigenic breast epithelial cell line MCF10a **c)**. The three cell lines were submitted to the different steps in the process to assess their suitability for performing the screen.

4.1.2 NKi Library

Immediately after receiving the pRetroSuper NKi shRNA library from the Bernards lab, I proceeded to package it into retroviral particles. Unfortunately, this turned not to be so trivial as in 8 out of 19 attempts to produce functional viral particles, the NKi shRNA library viral titre was either extremely low or non existent when tested on different cell lines(Figure 8a)). An aliquot of the library was submitted to both sequence verification and restriction enzyme digest to confirm the identity (Figure 8b). The only unexpected result was an unidentified but persistent (n=3) band at ∼6kb, which could either be due to incomplete digestion of the plasmid or due to a plasmid contamination. Given that the problems persisted we pursued to retransform the shRNA library into ultra-competent E.Coli bacteria.

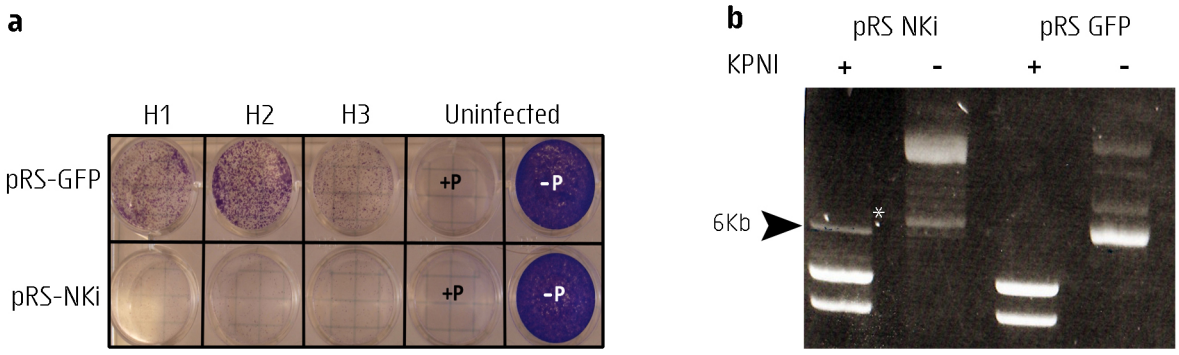

Figure 8: *Low viral titre with the newly acquired NKi library.* **a)** Crystal violet staining of U2OS cells infected with three different viral harvests (H1-H3) from the NKi library and the pRS-GFP vectors. Staining was done after two days of puromycin selection. **b)** Agarose gel electrophoresis pictures of the pRS-NKi and pRS-GFP vectors digested with the KPNI restriction enzyme.

4.1.3 BrdU/Hoechst/UVa

The method for killing dividing cells has previously been described[41, 8]. I attempted to establish killing protocols for all three candidate cell lines. Concentrations were left the same as had been described before, changing only the duration of the BrdU treatment. For our screen, survival after BrdU/Hoechst/UVa treatment should be reduced to roughly 2-4 cells per well of a multi-well 6-well well or 6-12 colonies on a 100mm dish so as to keep the background levels to a minimum.

First to test whether our experimental setup was correct, I attempted to replicate the experiment by Burmer and Norwood in HeLA cells[41]. As reported, after only a three day BrdU treatment, followed by Hoechst 33258 and UVa, 1-2 background colonies of HeLA cells were observed (Figure 9a). Having established the proper killing protocol

for HeLA cells, I proceeded to investigate whether similar results could be achieved in IMR90, MCF10a and U2OS cells.

4.1.3.1 IMR90 Being a primary fibroblast cell line, IMR90 cells have a rather intact karyotype compared to immortalised cell lines which can be quite heterogenous even within a certain culture. Previously it had been shown in several different skin fibroblast cell lines and specifically in IMR90 cell lines that a small population of slowly growing cells were resistant to the BrdU/Hoechst/UVa protocol probably due to low levels of BrdU incorporation[41, 43, 44]. As a matter of fact, after Hoechst 33258 and UVa irradiation only a small percentage of cells actually died (Figure 9b) and furthermore, IMR90 PD 14 cells treated with BrdU displayed a noticeable growth defect phenotype (Figure 9c). Therefore, the cell line was deemed unsuitable.

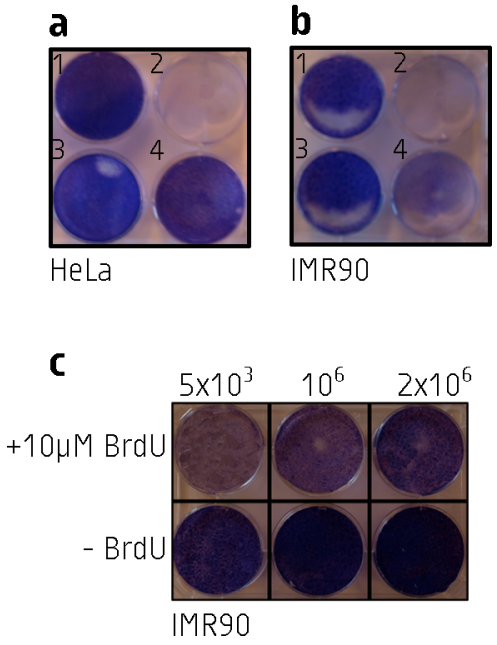

Figure 9: *BrdU Hoechst UVa treatment effectively kills HeLA but not IMR90 cells.* **a)** HeLa cells treated with both BrdU and Hoechst succumbed after UVa irradiation *2* in comparison to cells treated only with Hoechst *1* only with BrdU *4*. Non-treated cells are shown as a comparison *3*. **b)** Same as in a) with IMR90 cells. **c)** IMR90 cells plated at increasing densities and treated with *upper lane* or without *lower lane* BrdU at the indicated concentration, cells were stained with crystal violet dye after 4 days in culture.

4.1.3.2 MCF10a I next attempted the killing protocol on the MCF10a cell line[45]. These cells are not known to go into a senescent state even after multiple passages. In contrast to the IMR90 cells, MCF10a cells treated with BrdU displayed a normal growth phenotype (data not shown), and indeed after Hoechst 33258 and UVa treatment, very few cells seemed to survive. However, cells that did survive the treatment readily went into cycle forming >15 colonies per 100mm dish in a period of 2 weeks post treatment (Figure 10a). I hypothesise that the patch-like growth of MCF10a cells might hamper cells closer to the center of a patch to divide possibly by contact inhibition, and thus to properly incorporate BrdU (Figure 7c). Once the dividing cells which lie on the outside of the patch have been killed, contact inhibition is abrogated and cells

which had stopped cycling can start again.

4.1.3.3 U2OS As expected, the killing protocol worked seamlessly for the fast dividing U2OS cell line. The slight disadvantage with using cell lines is that they have been in culture for long periods (e.g U2OS since 1964) and there are doubts as to whether there is a resemblance to primary cancer cells. The advantage being that finding a gene that hinders such a sturdy cell line from growing could possibly prove extremely useful in cancer research. After a 72 hour BrdU treatment, $\leq$0.1% of cells formed colonies 2 weeks post treatment (Figure 10b). This made the U2OS cells the only viable candidate to perform a test screen.

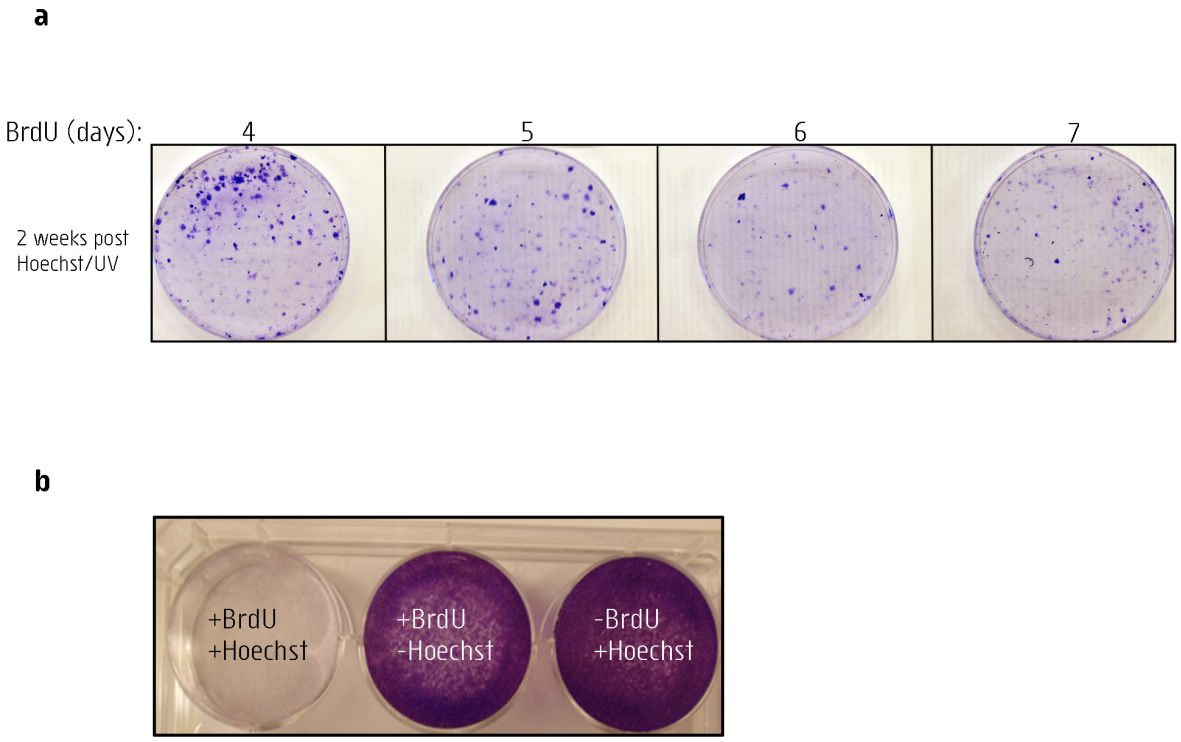

Figure 10: *BrdU killing protocol kills U2OS cells but not MCF10a.* **a)** MCF10a cells treated for different time periods with BrdU display an increasing mortality rate which correlates with the length of the treatment, however multiple colonies arise after the dishes are kept in culture for a 2 week period. **b)**U2OS cells treated only for three days with BrdU display none to two colonies (data not shown) 2 week after UVa irradiation.

4.1.4 LargeT antigen

I encountered problems both producing viable virus and sequence verifying an existing lentiviral pWPI SV40 Large T vector. Western blot analysis showed that in fact no Large

T protein was being made by the aforementioned vector. A second retroviral vector pBABE Large T did properly express the Large T antigen (Figure 11a). A retroviral vector is of no use to introduce the LargeT antigen into non-dividing cells to reinitiate S-Phase DNA synthesis. The options left are to either transfect the cells with the pBABE vector, or to shuttle the coding sequence for the Large T antigen into a lentiviral vector. Thus, we opted for shuttling the Large T coding sequence from the pBABE-hygro to a pLKO.2-CMV-puro vector. The finished vector was sequence verified and expression of the Large T antigen was confirmed by western blot analysis (Figure 11b).

Next it was important to determine whether Large T expression would reinitiate cell cycle in arrested cells. IMR90 cells go into a senescent state after hRASV12 expression in a p53/pRB dependant manner and this effect can be countered by expression of the SV40 Large T antigen[46, 47]. Thus we infected IMR90 cells with a lentivirus containing the RASV12 protein under a CMV promoter and after the strong senescent phenotype was observed in these cells, a subsequent infection with the plKO.2-CMV-LargeT virus was done. Large T infected cells were observed to go back into cycle and started forming colonies (Figure 11c) as opposed to non-infected cells, thereby confirming the functional expression of the Large T antigen.

As opposed to the pRS-NKI library virus, all three candidate cell lines were readily infected with the plKO.2-CMV-LargeT virus (data not shown).

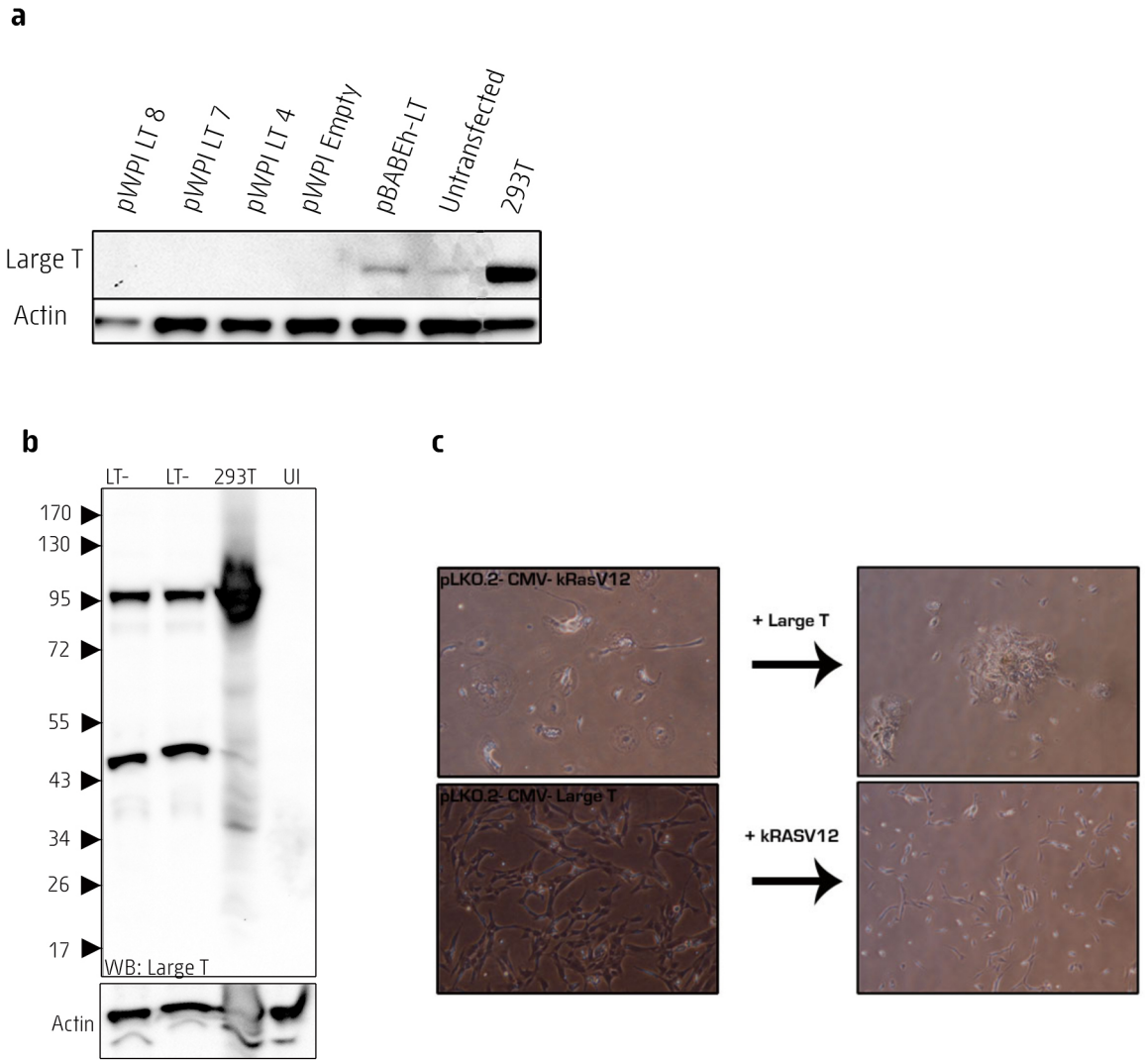

Figure 11: *Creation of a functional lentiviral vector expressing the SV40 Large T antigen.* **a)** Large T antigen could not be detected from the pWPI-Large T vectors when transfected into U2OS cells (lanes 1-3), the pBABE-Hygro Large T vector available did display a large T band at the expected size (lane 5). Untransfected 293T cell lysate was used as a positive control (lane 7) and untransfected U2OS cells were used as a negative control (lanes 3 and 6). **b)** The Large T sequence was shuttled from the pBABE-Hygro vector into a pLKO.2-CMV-Puro vector and U2OS cells were infected with the resulting virus. Two prominent bands at 97 and 45 kDa were detected for Large T and Small T respectively (lanes 1-2) untransfected 293T cells (lane 3) and untransfected U2OS cells (lane 4) were used as a positive and negative controls respectively. **c)** IMR90 transduced with a pLKO.2-CMV-kRASV12 expressing vector, and after 2 days infected with the newly created pLKO.2-CMV-LT vector went back into cycle (upper pictures). IMR90 cells transduced first with the pLKO.2-CMV-LT vector and then with the pLKO.2-CMV-kRASV12 vector did not go into cell cycle arrest and continued dividing (lower pictures).

4.2 Test screen in U2OS cells

After carefully considering the results from the previous section we came to the conclusion that if we were to make a test run for the screen, it would be advisable to use the U2OS cell line as a model. In summary, three important facts make this cell line an adequate model to work with. First, the U2OS cell line is reportedly p53/RB wild-type (although we did not test this assertion for our specific batch), second, high titre infections with both our pRetroSuper and pLKO.2 vectors were achieved and third the BrdU killing protocol killed most if not all dividing cells after only 72 hour treatment.

4.2.1 Layout and implementation of a test screen

The exact protocol followed is described in the *Materials and Methods* section *6.6*. U2OS cells were infected with both the NKi library virus and the pRS-GFP as a negative control. Cells were then submitted to the BrDU killing protocol and after a recovery period of 48 hours cells were infected with the pLKO.2-CMV-Large T lentivirus. At first, surviving cell numbers were indistinguishable between the NKi and the GFP infected U2OS cells. After a two week recovery period, two and three colonies were seen in each of the pRS-GFP negative control cells infected with the Large T lentivirus, whereas only one colony was observed in a control dish without Large T. This observation was in contrast with an average of five colonies per 100mm dish observed in the NKi infected U2OS cells (n=20 dishes. Total colonies collected = 100). Colonies were collected and separated to individual wells on a 48-well plate, identification of the candidates through genomic DNA isolation and subsequent PCR was done for each candidate as soon as enough cell material was available. We reasoned that a confluent 6-well would be more than sufficient for several tests, thus cells were carefully passaged from a 48-well to a 24-well to eventually a 6-well.

Table 1: Preliminary list of candidates which could be successfully sequence verified.

Gene	**Function**
NUCKS1 (nuclear casein kinase and cyclin-dependent kinase substrate 1)	Contains a putative DNA binding domain; and serves as a CDK1 Substrate.[48, 49]
ATR (Ataxia telangiectasia and Rad3 related)	ATR is needed for the activation of the S-phase checkpoint after UV induced ICL (DNA inter-strand link)lesions. Cells with siRNA against ATR will ignore lesions and go into cycle. [50, 51]
CRYM (Crystallin μ)	CRYM expression is androgen regulated in prostate cancer. [52]
PIP5KIß (phosphatidylinositol-4-phosphate 5-kinase; type I; beta)	Very large family. Diverse functions. [53]
LPXN (Leupaxin)	Expressed in human prostate carcinomas (Pca). Knockdown reduces migratory and invasive behaviour in PCa cells [54]

PSAP (Prosaposin)	Stimulates p53 expression in Pcas and inhibits tumour metastasis. [55]
FGFR3 (Fibroblast Growth Factor Receptor 3)	Rhabdomyosarcoma cells expressing FGFR3 have tumour initiating potential, and chemical inhibition of FGFR3 can induce apoptosis in myelomas. [56, 57]
CRK (Chicken tumour virus No.10 Regulator of Kinase)	Originally described as a viral oncoprotein CRK is downstream of FGFR signalling.[58, 59]
CD68	afjkaef
BID (BH3 Interacting Domain Death Agonist)	Over-expression of BID induces P53 independent apoptosis. BID levels are regulated by p53, BID is downstream of p53. [60, 61, 62]

4.2.2 Preliminary candidates

Out of the 100 colonies originally collected 55 of the candidate colonies did not survive after being passaged and transferred to individual wells. Nonetheless, surviving candidate colonies were collected, the individual genomic DNAs isolated and sequence verified (Table 1). Once again, only 21 of the 45 genomic DNAs analysed seemed to contain a 19-mere insert, indicating to a rather high background level.

The candidates that could be sequence verified were then shuttled into a pRetroSuper vector for individual confirmation of the cell cycle arrest inducing phenotype. The protocol followed was the same as with the test screen. No morphological changes were observed after infection with any of the hairpins, however after BrdU selection, U2OS cells infected with five hairpins seemed to undergo cell cycle arrest as assessed by morphological changes of the cells through light microscopy(Figure 12). To test whether infection with the Large T lentivirus would bring the cells back into cycle, I proceeded to passage them to be able to do the infection in duplicate. Unfortunately, this resulted in cell death of all the candidates.

Subsequent multiple attempts to reproduce this experiment were unsuccessful due to technical reasons. Nonetheless I proceeded to evaluate the knockdown efficiency in order to see that this was not just an off-target effect.

4.2.2.1 RT-PCR and Western blot confirmation of knockdowns A common problem encountered when working with shRNAs is that the desired phenotype is elicited not by a biological effect of the knockdown but by an off-target effect of the hairpin. The shRNA hairpins contained in the NKi library have neither been sequence verified

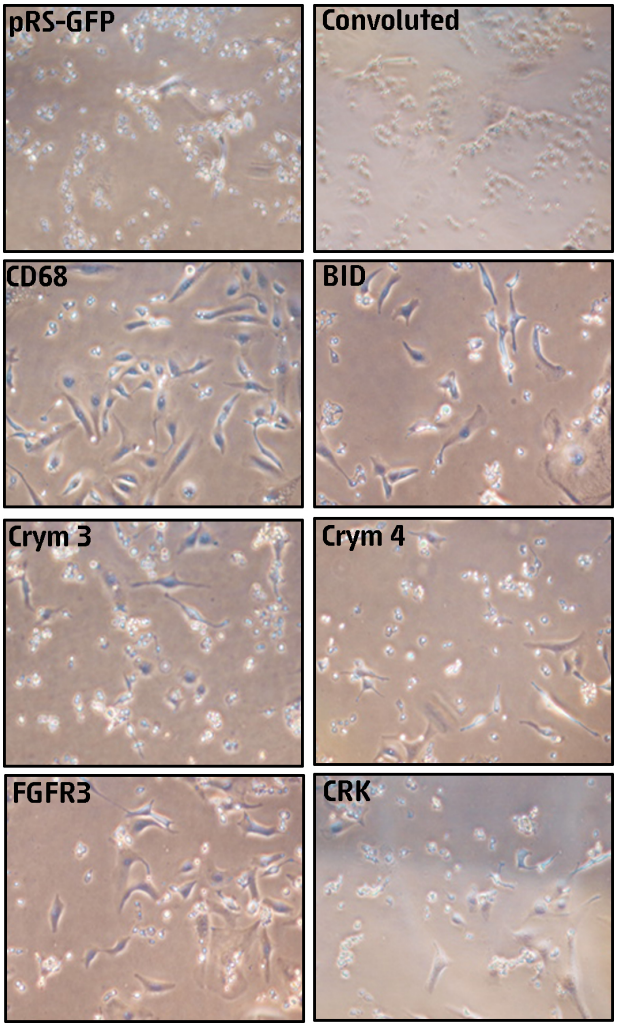

Figure 12: *shRNAs found to induce a cell cycle arrest in U2OS cells.* U2OS cells infected with retroviral vectors containing an shRNA targeting the indicated genes and submitted to a BrdU Hoechst UVa treatment. Cells infected with a pRS-GFP vector or with a vector which could not be identified from the screen all died after treatment. A total of 17 vectors were successfully shuttled from the genomic DNA of U2OS cells from the screen into a pRetro Super vector and out of these, five seemed to induce a senescent phenotype. Cells were infected with the vector containing the CRYM shRNA in 4-plicate and all displayed a similar phenotype.

nor the biological knockdown efficiencies have been tested. Thus, it is critical when working with the NKi library that at least the latter is confirmed before proceeding to validate a candidate. For this I analysed the knockdown levels of candidate genes that purportedly induced an arrest. This was done with a RT PCR reaction for all candidates and WesternBlot analysis for CRK and FGFR3. Primer sequence design and antibodies used are described in the Materials and Methods (sections 6.7 and 6.12).

Decreased mRNA levels could be observed in all samples except BID (Figure 13a and data not shown for BID), although the standard deviation from three biological replicates with the CRYM and CD68 hairpins was too high to be able to rule out an off-target effect.

The knockdown of CRK and FGFR3 aroused our interest since both have been previously reported to be involved in the same signalling pathway, as was shown with the tyrosine phosphorylation of Crk mediated by the interaction between FGFR1 and the SH2 domain of Crk [59]. Western blot analysis of whole cell lysates was performed with cells transduced with either the Crk or the FGFR3 shRNA. There is a clear reduction

of the FGFR3 protein levels whereas there does not seem to be a reduction in the Crk levels (Figure 13b).

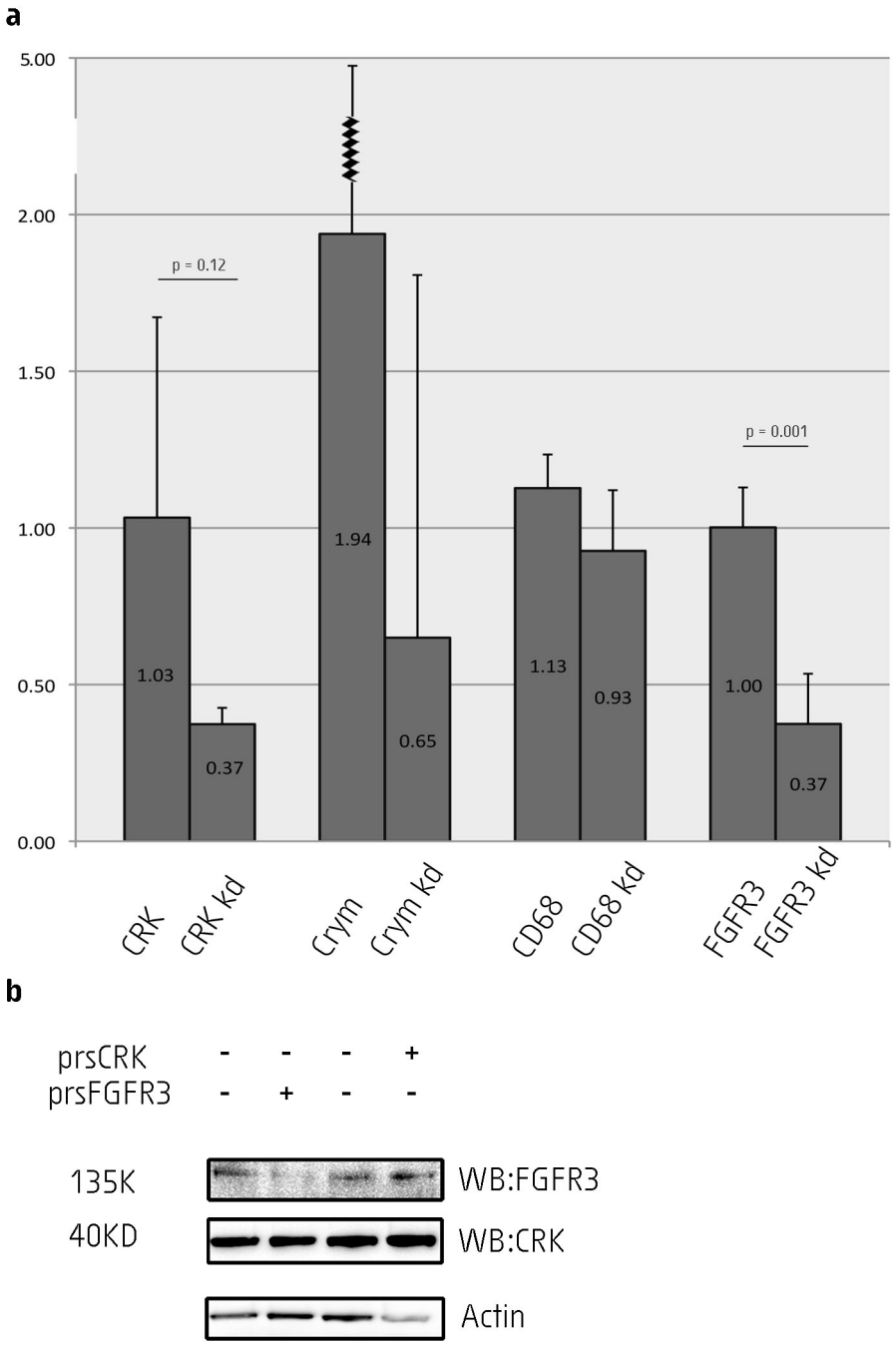

Figure 13: *mRNA and Protein levels of candidate genes after transduction with the candidate knockdown vectors.***a)** Relative change of mRNA levels of U2OS cells transduced with the indicated knockdown vectors normalised to U2OS cells transduced with a pRetroSuper-GFP control vector. $\Delta\Delta$-RT values. Error bars indicate standard deviation of three separate biological replicates.**b)**Western Blot analysis of U2OS whole cell lysates transduced with the indicated hairpin or with the pRS-GFP control vector.

4.2.3 FGFR3 and CRK

Both FGFR3 and Crk proteins have been shown to have oncogenic potential. Multiple oncogenic properties of Crk have been extensively described (reviewed in [63]). Mostly, effects of Crk knockdown in tumours decreases their metastatic and invasiveness potential. Although, it has been shown in Bcr-Abl positive leukemic k562 cells that the disruption of the CrkL - Bcr-Abl complex strongly inhibits cell proliferation[64], not many other proliferation related effects have been shown in the absence of Crk.

A search with the oncomine database revealed that FGFR3 is significantly over-expressed in primary osteosarcoma tissue samples (Figure 14). Several groups have published data that assign tumourigenic properties to FGFR3[65, 56]. Furthermore, one group in particular seemed to be making an association between osteosarcoma and FGFR3 [66], thus I attempted to reproduce some of the published experiments in U2OS cells.

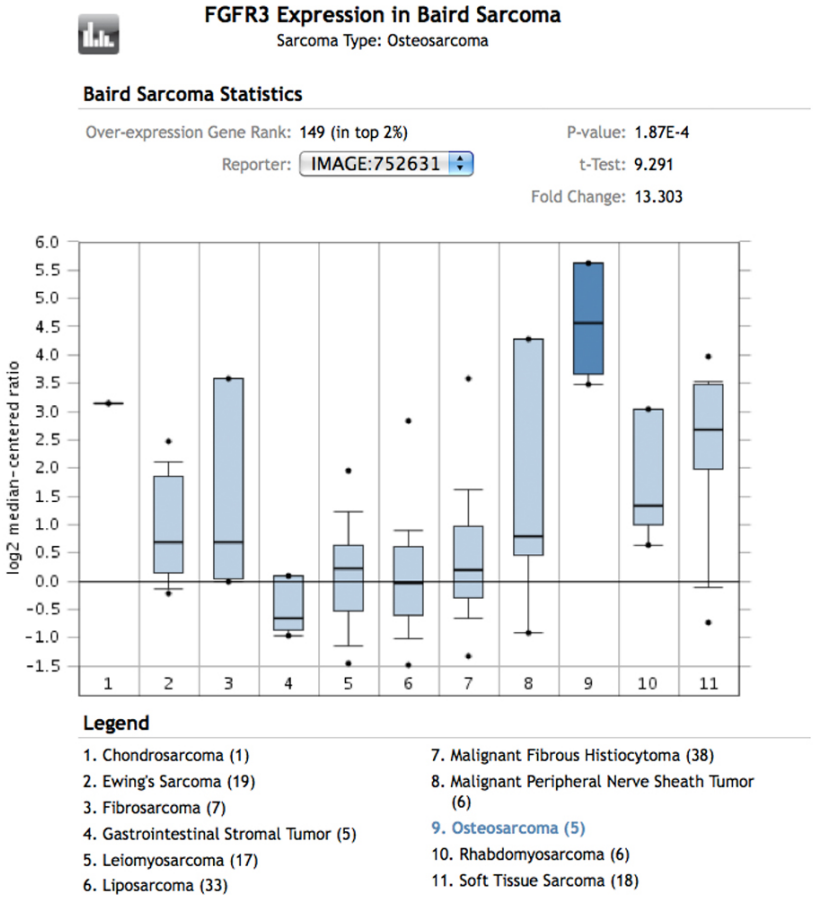

Figure 14: *FGFR3 is over-expressed in Osteosarcoma samples.* The Oncomine sample database analysis found a significant over-expression of the FGFR3 protein in Osteosarcomas as compared to ten other sarcoma types.

4.2.4 FGFR3 Inhibitor treatment of U2OS cells

Several signaling pathways leading to and from FGFR3 have been extensively studied [65]. In our case, we decided that the next step was to see whether we could mimic the cell cycle arrest seen with the knockdown vector by chemically inhibiting FGFR3. We approached this question with two inhibitors which have specifically been shown to target the FGFR3 receptor in vivo: PD173074 and Masatinib.

The PD173074 compound selectively inhibits the tyrosine kinase domain of all FGFR

receptors at a concentration of ≈25nM [67, 68]. PD173074 was also shown to be biologically active and induce FGFR3 dependent apoptosis in myeloma and inhibiting growth in a mouse xenograft model [57]. The second compound we used is Masatinib another tyrosine kinase inhibitor which is currently in Phase III clinical study for treatment of Gastrointestinal stromal tumours [69].

I tested the viability of three different osteosarcoma cell lines treated with either one of the tyrosine kinase inhibitors, however a significant decrease in viability was only reached at physiologically very high concentrations of $>3\mu$M (Data not shown). Thus, treatment of osteosarcoma cell lines with FGFR3 inhibitor was deemed insufficient to cause cell cycle arrest or apoptosis in these cell lines. The actual physiological activities of the FGFR3 inhibitors was not tested to to time and technical constrains.

4.2.5 Subpopulation of FGFR3 positive U2OS cells

The theory that cancers might possess the ability to renew themselves by a subset of tumour initiating cells (TICs) has grown in credibility in recent years [70]. If the presence of these cells turn out to be a general mechanism of cancer survival, finding specific cellular markers could proof to be a powerful new asset in the quest to defeat this disease. Recently published work demonstrated that in a population of rhabdomyosarcoma cells, roughly 1-3% of the cells were found to express FGFR3. Incidentally, FGFR3+ cells were more prone to elicit tumour formation when inoculated into nude mice than FGFR3- cells[56].

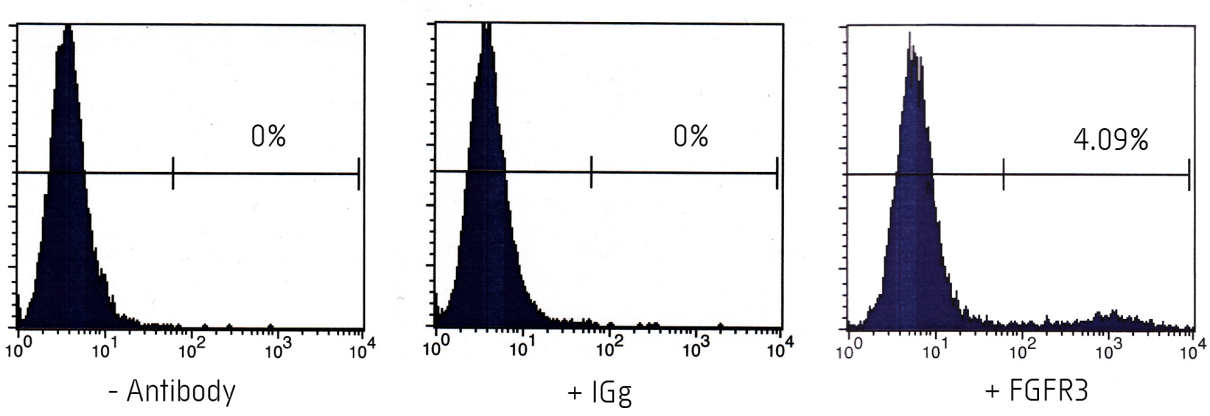

Figure 15: *U2OS cell line has a small population of FGFR3 expressing cells* U2OS cells shown are either untreated, labelled with a IgG isotype antibody or a PE-conjugated FGFR3 antibody and then analysed by flow cytometry. Populations of FGFR3 expressing cells ranged from 2-9% in three separate experiments.

One way to explain our previous results (section 4.2.4) was the possibility that in our screen (section 4.2.1) the U2OS cell originally underwent cell cycle arrest could have

been a TIC which was dependent on FGFR3 signals to divide. An arrest at a whole cell population level through the inhibition of FGFR3 either by a knockdown vector or by a chemical inhibitor would thus be only detectable after a prolonged period of time.

To verify our hypothesis, we attempted to first determine whether as in rhabdomyosarcoma cells, a small FGFR3+ population could be detected. Fluorescent activated cell sorting (FACS) analysis, seemed to indeed point to a small subpopulation of FGFR3+ cells residing within a wild-type U2OS population (Figure 15).

Next, I investigated whether the inhibition of FGFR3 would have an effect in the overall growth of U2OS cells kept in culture for a long term. For this I transduced one batch of U2OS cells with the FGFR3 shRNA lentiviral vector and kept a second batch under constant treatment with the PD173074 inhibitor counting the cell numbers every two days. The expected long term decrease in cell number was not observed after a two week period and the experiment was thus terminated (data not shown).

Further attempts to verify the effects of the FGFR3 knockdown were inconclusive. Most importantly, the subpopulation of FGFR3+ cells observed in the FACS experiment, did not disappear in the FGFR3 shRNA transduced cells.

4.2.6 Mycoplasma infection

Throughout the follow up of CRK and FGFR3 many attempts were done in parallel to reproduce the effects seen in 4.2.2. Unfortunately this did not turn to be as straightforward as expected. Results varied greatly after each BrdU / Hoechst / UVa treatment, with sometimes treated cells surviving the whole treatment altogether. Even after extensive reagent tests where each of the three components were tested separately, no likely explanation could be found to answer the dilemma. Through literature research, I stumbled upon an article on the webpage for the German Collection of Microorganisms and Cell Cultures which stated that "in the presence of mycoplasmas, BrdU is degraded and the eukaryotic cells survive even though they do not possess a TK defect"[71]. Indeed it appears that a combined treatment of BrdU and Light had been previously used to eliminate *Mycoplasma Orale* from cell lines kept in culture[72]. It appears as if mycoplasmas might insert BrdU into their genomes as well, killing them in the process.

Alas, a quick test with the mycoplasma Kit from R&D systems proved the mycoplasma hypothesis to be right. It would seem that Mycoplasms insert the BrdU into their own genomes and the human cell cultures are left with none to insert. If there was a significant amount of mycoplasmas on the dish, then most of the BrdU would be absorbed in the process and the cell cultures would not die. on the other hand if there were only few

mycoplasmas on the dish upon start of the BrdU treatment, then cell cultures would die, albeit to a much lesser degree as non-mycoplasma infected cells.

After a two week treatment of cell cultures with the MycoKill antibiotic, a new definitive series of tests to confirm the effects seen in Figure 12 was conducted. Again, U2OS cells were infected with the candidate shRNA lentiviral hairpins and submitted to the BrdU/Hoechst/UVa killing protocol (Fig 16). Unfortunately, this test done in duplicate disproved our results, as all cells perished after the treatment. Interestingly, this time we infected the cells with a hairpin targeting the HDM2 mRNA sequence as a positive control. Cells with low levels of HDM2 were expected to go into p53 mediated cell cycle arrest or apoptosis due to elevated p53 levels, and should be readily rescued via the introduction of the Large T antigen. Although more colonies do grow out from the U2OS cells infected with the pRS-HDM2, there is no obvious difference between cells infected with the Large T vector versus untreated cells (Fig 16).

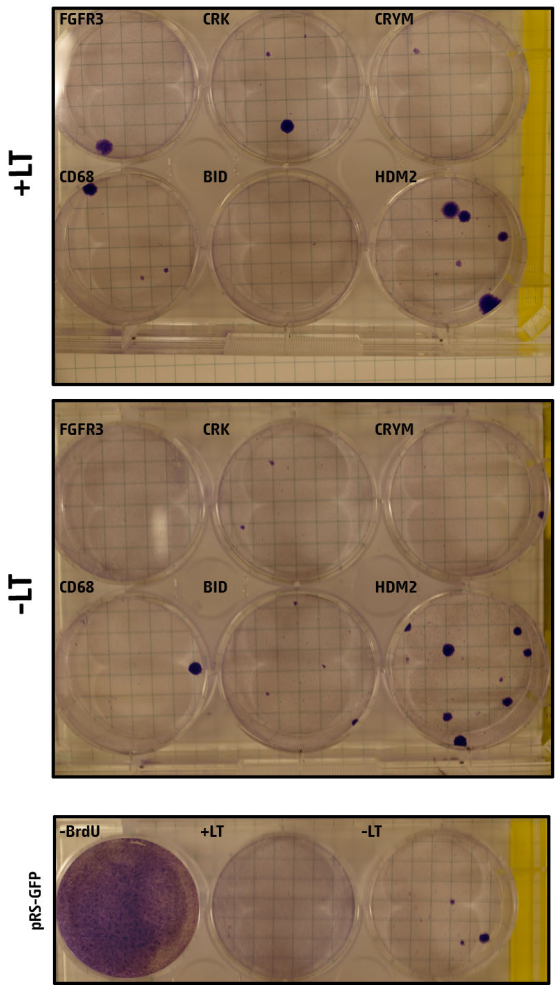

Figure 16: *shRNAs found to not induce a cell cycle arrest in U2OS cells.* U2OS cells infected with retroviral vectors containing an shRNA targeting the indicated genes and submitted to a BrdU Hoechst UVa treatment. After a 48 hour recovery period, cells were either infected with the pLKO.2-CMV-Large T vector (upper panel) or left untreated (middle panel). Cells were stained with crystal violet after a 20 day recovery period. Cells were infected with a pRS-GFP vector as a negative control (lower panel) and submitted to the same treatment.

Given that most of the follow up experiments on CRK and FGFR3 had been started already we decided to carry on albeit the discouraging results.

5 Discussion

Every new technology is bound to meet setbacks before being fully implementable. As expected at the beginning of my yearlong project, the setting up of a new screening method from scratch would prove challenging. Recently the group of Wei Gu described the previously uncharacterised protein ULF to be a ubiquitin ligase for ARF[73]. Interestingly, knockdown of ULF caused the stabilisation of ARF in primary human cells triggering ARF-dependant p53 cell cycle arrest. The discovery of ULF showed that unknown inhibitors of p53 activators remain to be found and that the cell actively uses this kind of signalling to counteract environmental insults. Thus, during my thesis I have successfully laid down the foundation for what could be a very promising tool. The efficacy of each of the four steps in our approach can hitherto be tested independently using other model cell lines.

5.1 Test Screen - Controls

Admittedly, there were some flaws in the first test screen I performed in U2OS cells (see section: 4.2.1). This made it impossible to assess with a strong confidence whether actual hits were found. Nonetheless, I am confident that there is a great potential for this screening method to positively detect further novel inhibitors of the pathways leading to cell cycle arrest. Given more time I would have repeated the U2OS screen taking into account several of the mistakes from the test screen and modifying these parameters accordingly.

First of all, the lack of appropriate positive and negative controls made it impossible to adequately determine the signal to noise ratio of our screen. A good positive control could give us the definitive proof that our approach works, however given our mishap with HDM2 (section 4.2.6) it will prove challenging to find an appropriate control. Instead we propose an alternative method by slightly altering the screen so as to include a test positive control within the screen itself. The idea being to follow the protocol as-is up to the Large T infection. At which point, instead of picking individual colonies, one would count all present colonies and then pool them together without ever passaging them. The genomic DNA can then be isolated from the pool of colonies, and a PCR reaction is performed to amplify all hairpins present. The PCR products can then be ligated into an empty pRetroSuper vector to create a new library NKi 2.0 theoretically enriched with positive hits. Whether this is true can be easily shown by performing a second screen with the new NKi 2.0 library. We hypothesise that if the BrdU killing protocol can indeed selectively kill dividing cells, then one would expect a much larger number

of colonies forming in a screen of the NKi 2.0 than with the original NKi 1.0 library. If this hypothesis holds, then the protocol is followed as described above (Section 4.2.1) to identify individual hairpin candidates.

As to the negative control, with our conditions it was impossible to determine the number of background colonies in untreated cells during our screen. We have observed a very low background level (1-2 colonies per dish) in independent experiments (Figures 10b and 16 and data not shown), however it is very important to assess this in parallel to the pRS-NKi infected cells. During my time at the lab, I did indeed start a series of new screens in which I had adjusted for this parameter. I attempted to repeat the screen in several occasions adjusting for this parameter by infecting a total of 6.0×10^7 cells with the NKI virus and a total of 2.0×10^7 cells with the control pRS-GFP vector. Unfortunately these attempts had to be stopped once the mycoplasma infection previously described was identified (section: 4.2.6).

5.2 General Improvements

Overall, with the experience I have gained, I have recognised several potential bottlenecks and given the time would make the following changes when performing a screen either on the U2OS cell line or on another cell system. Again, I cannot emphasise enough that the most important method is the killing protocol, however once this hurdle is overcome, and an appropriate cell line has been found there are certain changes which would make the screening method more robust.

5.2.1 Stable inducible expression of the LargeT antigen

We chose to deliver the Large T antigen into non-dividing cells by Lentiviral infection. It is the most reliable if not the only method for delivering a foreign DNA sequence into a non-dividing cell's DNA. When following our experimental conditions at the time of the LargeT - dependent cell cycle induction, cells have been exposed to several stresses namely: Infection with a retroviral vector, induced cell cycle arrest, BrdU and subsequent UVa treatment. Furthermore, if the cell has been brought to the cell cycle arrest stadium in a p53 dependant manner, its DNA-repair capabilities might be compromised and insertion of the lentiviral DNA might produce irreparable damage. Thus we believe that an inducible expression system would increase the signal to noise ratio of our screen. We propose that once the appropriate cell line is found, the cell line can be modified to stably contain and express the Large T antigen under the control of a Tet-On tetracycline inducible promoter [74]. This way the LargeT expression can be activated from within the cell without tampering further with the genomic make up.

5.2.2 Alternative Screens

Of particular interest would be to attempt the screening method on different cell lines, specifically applying the method on primary tumour cells or a primary non-tumorigenic cell line. These cell lines are of much closer genetic resemblance to human and thus serve as a better model. Thus, the results arising from such a screen would be of a greater clinical value. As I have shown, during the first half of my thesis I have attempted to establish some of the screening steps with different cell lines (section 4.1.1). Several setbacks were encountered with each of the cell lines however I am confident that given more time, an appropriate cell system will be found. Although all steps in the screen are important in themselves, the most critical step remains the BrdU/Hoechst/UVa killing protocol. In order to reduce background it is of upmost importance to achieve a killing degree comparable to that achieved in the U2OS or HeLa cell lines (Figures 9 and 16).

5.2.3 Variations of the screen

Cells lines which are deemed suitable for the BrdU killing procedure can be screened in different ways. For example newer, larger, sequence and functionally verified shRNA libraries like the ones created by The RNAi Consortium (TRC) can be used.

Furthermore, in addition to performing the screen with the Large T antigen as a means to induce arrested cells back into cycle, the screening method could also be expanded to use other known oncoproteins. There is a myriad of proteins which would come into question, a good candidate would be the human papilloma virus (HPV) E7 oncoprotein which has been shown to be required for HeLa cell proliferation in the presence of the HPV E6/E7 inhibitor protein E2 in a pRB dependant manner [75]. Thus, a further application of the screening method can be conceived that uses the E7 protein instead of the Large T antigen to bring cells back into cycle to gain further insight into the pathways which lead to cellular senescence.

5.3 Candidate Follow up

The effect of FGFR3 knockdown on cell proliferation is definitely an area of interest (see [65, 56, 66] or Figure14), none of these studies have provided insight into the mechanistic properties of the observed oncogenic potential of FGFGR3, thus a link to the p53 pathway can neither be assumed nor entirely ruled out. Although the hit could not be confirmed in our hands it is still plausible that we have found it as a positive hit with our screen. There exists a vague possibility that we knocked down FGFR3 in the subpopulation of

FGFR3 expressing cells (Figure 15) and that this caused cell cycle arrest. It would be interest to try to isolate these FGFR3 cells through flow cytometry and see if knockdown of FGFR3 in these cells will induce the expected cell cycle arrest.

Looking at the RT-PCR data, we decided to discard the rest of the candidates as background noise as the shRNA vectors seem to induce no visible knockdown of their target genes. Although given the variance in some of the samples (i.e. CRYM in Figure 13a) it would also be advisable to measure the mRNA levels again with different RT PCR primers .

6 Materials and Methods

6.1 Cell lines

IMR90 (ATCC ID: CCL-186) fibroblasts cells are derived from a human embryonic lung of a 16 week female foetus. The cells were taken in culture at passage 13. Attempts to immortalise them with a hTERT lentiviral construct were unsuccessful.
U2OS (ATCC ID: HTB-96) osteosarcoma cells are derived from a sarcoma of the tibia of a 15 year old girl. These cells are p53 and pRB wild-type. No known viral proteins have been detected.
HeLa (ATCC ID: CCL-2) cells are derived from a 31 year old female and are widely used in research. HeLa cells have been reported to contain HPV-18 sequences.
MCF-10a (ATCC ID: CRL-10317) cells are derived from from a non-tumorigenic epithelial cell line of a 36 year old female. The cells exhibit three dimensional growth in collagen. No known viral proteins have been detected.

6.2 Vectors

6.3 Primers

6.3.1 Large T Cloning

Forward primer with AgeI site: GATCCCACCGGTACCATGGATAAAGTTTTAA Reverse primer with EcoRI site: GATCCGAATTCTTATGTTTCAGGTTCAGG

The primers were designed to amplify the LargeT sequence from a functional pBABE-hygro-CMV-LargeT. A PCR was performed using 10ng of the pBABE-hygro vector, the product was then digested with EcoRI/AgeI restriction enzymes and ligated into a EcoRI/AgeI digested pLKO.2-CMV-Puro vector. XL10 Gold E.Coli cells were transformed with the ligation product and grown on LB Amp- selective plates. Plasmid DNA was then isolated from growing E.Coli colonies. Plasmids were sequence verified and functional expression of the Large T antigen was verified through Western Blot analysis.

6.4 BigDye© Sequencing reaction

The BigDye sequencing reaction was done with the Qiagen BigDye© Terminator v3.1 sequencing kit. It's based on the linear amplification of a DNA sequence. Per standard sequencing reaction, 50-100ng PCR product or 250ng plasmid DNA were mixed with 1μl

5x sequencing buffer, 1μl primer (use only one primer per reaction), and the 0.5μl of the BigDyev3.1 master mix. The mix was filled up to 10μl with MQ and incubated on a thermocycler instrument with the following program: 96°C for 1', 96°C for 10", 50°C for 5", 60°C for 4' repeat 25 times.

To purify the LAMP product from non-incorporated nucleotides, 10 μl MQ were added, the mix was eluted over a G50 Sephadex minicolumn at 2200g for 2 minutes. 2μl of the purified product were mixed with 10μl Formaldehyde.

6.5 Virus Production in 6-well plate format

HBS
$CaCl_2$

6.5.1 Virus production

Final concentrations of DNA are not important for the transfection. The quantities mentioned here are for transfections of 1 well of 70-80% confluent 293T producer cells on a 6-Well plate. 2.3 μg δR8.91, 0..3μg VSVG, 0.2μg CMV-GFP were mixed in one tube and 0.4μg of the lentiviral vector of interest were added to the mix. Then 158μl 1X HBS pH6.5 was pipetted to the DNA mix, and as quickly as possible 8.33μl $CaCl_2$ are added to the solution. The HBS/DNA/$CaCl_2$ mix was vortexed immediately. 293T packaging cells were plated for no longer than 12 hours prior to the transfection. The transfection mix was pipetted drop-wise directly onto the medium. After an incubation of ≈24 hours the 293T cells are checked under the fluorescent microscope, and I proceeded to the next step only when 90% of cells were GFP positive (green).

The DMEM was changed with 1.5mL fresh DMEM. DMEM supernatant containing the viral particles was harvested in roughly 12-14 hour intervals, but never more than three times. To remove remaining 293T cells from the medium, the supernatant was either spun down at full speed for 5 min, or filtered through a 45μM sterile syringe filter.

6.5.2 Test infection

To determine virus titre, harvested supernatant was applied directly to target cells in a dilution series. For this a master mix of 10X polybrene and the viral harvest filled up to 1ml with DMEM was prepared. Infection efficiency was validated either by selecting with an antibiotic or by fluorescence microscopy if a GFP tag is present in the vector of interest.

6.6 Test screen in U2OS cells

To achieve an infection rate with a high confidence of hitting all vectors at least once we would have to infect 4.8×10^6 cells. For an even higher confidence we decided to infect three times as many cells thus we infected 13.5×10^6 cells. As a negative control I infected 2.5×10^6 cells with a pRetroSuper vector containing an antisense GFP shRNA hairpin. The BrdU (10μM final concentration) treatment was started after selecting for 48 hours on puromycin and was left on for 72 hours. To avoid overgrowth, and resulting contact inhibition dependent growth arrest, the dishes were split once they reached a 70% confluency. The cells were then treated with Hoechst #33158 dye (10μg/ml final concentration) for 3 hours and the cells were then irradiated from the bottom with a 230V, 5 Watt E27 UV lamp for 15 minutes after which fresh medium was added. The cells were infected with the plKO.2-CMV-Large T virus 48 hours after UVa irradiation, a control dish with untreated U2OS cells was infected to assess virus titre. The dishes were kept in culture for three weeks, changing the medium every 3-4 days. The dishes were washed once with PBS after which 7mL PBS was added per dish. A total of 100 colonies were picked with a 200 μl Gilson pipette and transferred to a 96-well plate containing 100μl of trypsin and incubated at 37°C for 4 minutes. The ∼150μl were then transferred to 48 well plates containing 700μl DMEM. Cells were allowed to grow to a confluent state at which point they were transferred to 6-well plates. Genomic DNA was isolated from confluent 6-well wells using the Promega Wizard Genomic DNA Purification Kit. A PCR using the fwd and rev primers was performed to amplify the inserted hairpin from the genomic DNA. The 200bp PCR products were sequenced to establish the identity of the inserted hairpins.

6.7 Western Blot

4x SDS sample buffer 320 mM Tris-HCl pH 6.8 40% (v/v) Glycerol 0.008g bromphenol blue 8% SDS (stock is 20%) 20% (v/v) beta-mercapto-ethanol (add freshly) Add all reagents to make 500 ml total but beta-mercaptoethanol Then final volume = 400ml

0.2% (w/v) iBlock solution 0.2g/100ml PBS iBlock powder Heat to 60°C in the microwave Stir until granules are dissolved Add Tween 20 (0.1% v/v)

Running Buffer Blotting Buffer ECL

6.7.1 Harvesting

Cells were infected or transfected with a plasmid containing our gene of interest (GOI). Cells were harvested after sufficient time for expression (usually two to three days). First

the cells were washed once with PBS and then 200-300μl 4x sample buffer were added. The cells were scraped from the dish with a rubber policeman and transfer into a 1.5ml eppendorf tube.The samples were boiled at 95°C for 5-30 min, depending on viscocity.

6.7.2 Running

After the samples have cooled down, 5-25μl (up to 20μg/ lane) were loaded on a pre-poured polyacrylamide gel. 4μl of the pre-stained marker were used each time. The current was set up to 200V for as long as required in running buffer (until front had reached the bottom of the gel). After the run,50% of the running buffer were always poured back into the original container and the rest was discarded.

6.7.3 Blotting

The gel was transferred into blotting buffer trying to always keep the membrane wet. Do not touch the membrane without gloves. To blot, a PVDF membrane was cut to the same size as the polyacrylamide gel. The membrane is activated by washing 1x 5min. in 100% ethanol. The membrane was placed on top of the gel and pressed together with the help of filter paper and sponges. The blotting reaction was run at 400mAmp keeping in mind that proteins are negatively charged and will mobilize in the direction of the cathode.

6.7.4 Antibody incubation and exposure

After the transfer, the protein ladder should be visible on the PVDF membrane. The membrane was incubated for 30min in BSA or iBlock blocking solution to avoid background detection of the antibodies and later was incubated at 4°C overnight with the appropriate primary antibody. The next day, the membrane is washed 3x 10min. with PBS-Tween and then incubated for approx. one hour with the corresponding PE conjugated secondary antibody. After incubation the membrane was washed 3x 5min. with PBS-Tween, then dipped 3x into a 1:1 ECL mix. Proceed to develop membrane.

6.8 Transformation of E.Coli Competent cells

For this, the E. coli strain XL10 Gold(galE galK galU+) is used. It has a doubling time of approx. 50 min. 50 μl of competent E. coli cells were thawed on ice and gently mixed with 5 μl of Ligation mix. The suspension was incubated for 10 minutes on ice. The cells are then heat shocked for 30 seconds at 42°C on a water bath and quickly put back on ice

for 2 minutes. 250 μl of SOC medium are added and the transformed cells are incubated at 37°C, 250rpm for 30 minutes. Cells were plated and incubated on selective lysogeny broth (LB) plates at 37°C over night.

6.9 DNA isolation

6.9.1 Minipreps

The day after transformations, single colonies were picked and grown over night in 4ml LB medium containing the Ampicilin antibiotic at 37 °C. Spin down 3 minutes at 6000 rpm the next day. The pelleted bacteria was resuspended in 250 μl buffer P1 and transferred to an Eppendorf tube. 250 μl buffer P2 are added and the tube was mixed thoroughly by inverting 4 - 6 times. 350 μl buffer N3 were added and mixed immediately and thoroughly by inverting the tube 4 - 6 times. The mix was centrifuged for 10 minutes at 13,200 rpm in a table-top micro-centrifuge. The supernatants were decanted onto the QIAprep spin column which was then centrifuged for 30 seconds. The QIAprep spin column was then washed once with 0.5 ml buffer PB and once with 0.75ml buffer PE. The QIAprep column was then placed in a clean 1.5 ml Eppendorf tube and to elute DNA, 50 μl water was added to the center of each QIAprep spin column.

6.9.2 Extract and purify DNA from standard agarose gels in TAE buffer:

For this, we used the QIAquick Gel Extraction Kit. Up to 400 mg agarose can be processed per spin column. The DNA fragments were excised from the agarose gel with a sterile, sharp scalpel. The gel slice was weighed in a colourless tube and 3 volumes of buffer QG were added per 1 volume of gel. The mix was incubated at 50 °C for 10 until the gel slice had completely dissolved. One gel volume of isopropanol was added to the sample and the mix was placed in a QIAquick spin column. The sample was applied to the QIAquick column, and centrifuged for 1 minute. After discarding the flow-through, 0.5 ml of Buffer QG were added to the QIAquick column and centrifuged for 1 minute. 0.75 ml of Buffer PE were added to the QIAquick column to wash and then it was centrifuged two times for 1 minute. 50 μl of water were added to the center of the QIAquick membrane and the column was centrifuged for 1 min.

6.9.3 Maxipreps

A single colony from a freshly streaked selective plate was picked with a pipette tip and 200ml LB containing the appropriate selective antibiotic were inoculated and incubated

for approx. 16 hours with vigorous shaking at 37°C. The bacterial cells were harvested by centrifugation at 6000x g for 15 min at 4°C. The bacterial pellet was resuspended in 10 ml Buffer P1 until no flocks are left. 10 ml Buffer P2 were added and mixed thoroughly by vigorously inverting the sealed tube 4-6 times, and incubated at room temperature for 5 minutes. 10 ml chilled Buffer P3 were added to the lysate, and mixed immediately and thoroughly by vigorously inverting 4-6 times. The lysate was poured into the barrel of the QIAfilter Cartridge and incubated at room temperature for 10 min. The plunger was gently inserted into the QIAfilter Maxi Cartridge to filter the cell lysate into a 50 ml tube. 2.5 ml Buffer ER were added to the filtered lysate, and it was mixed by inverting the tube approximately 10 times and incubate on ice for 30 min. A QIAGEN-tip 500 by was in the meantime equilibrated by applying 10 ml Buffer QBT, thus allowing the column to empty by gravity flow. The filtered lysate was then added to the QIAGEN-tip and allow it to enter the resin by gravity flow. After the lysate had flowed trough the QIAGEN-tip was washed 2x with 30 ml Buffer QC. The DNA is eluted with 15 ml Buffer QN and collected in a tube pretreated with isopropanol. To precipitate the DNA 10.5ml (0.7 volumes) room-temperature isopropanol were added to the eluted DNA mixing and centrifuging immediately at 15,000 x g for 30 min at 4°C after which the supernatant was carefully decanted. The DNA pellet was washed with 5 ml of endotoxin-free room temperature 70% ethanol and centrifuged at $>$ 15,000 x g for 10 min. Decant supernatant. The pellet was resuspended in 180 μl ddH2O.

6.10 Dephosphorylation

20 μl of digested (and in this case blunted) DNA was mixed with 3 μl of 10x CIAP buffer and 3 μl of CIAP and ddH2O was added to a final volume of 30 μl. This reaction mix was incubated at 37 °C for 30 min. Afterwards, the enzyme was inactivated by heating to 65°C for 15 min and the DNA was purified for ligation using a Qiagen PCR purification kit according to the manufacturer's protocol.

6.11 Ligation

For ligation 2.5 μl of 2x ligation buffer is mixed with appropriate amounts of vector and insert DNA and 0.5 μl T4 DNA ligase (3 u/μl, Promega), giving a final volume of 10 μl. After incubation for 3 h at RT or overnight at 16°C, this mix is used for transformation.

6.12 Real-Time PCR

6.12.1 Primers

FGFR3
Forward primer:
GTCGGCGCCTTTCGAGCAGT
Reverse primer:
CATGCCGGGGAGTGGCTGTG
Product size: 211
CRK
Forward primer:
TCTCAGGCAGTGCAAATCAC
Reverse primer:
CGGTGCATGATTACTTCACG
Product size: 162
BID
Forward primer:
AGCTGCTGCAGGCCTACCCT
Reverse primer:
AAGGACGGCGTGTGACTGGC Product size: 100bp
CRYM
Forward primer:
GGATCGCGGTGCGGAGACTG
Reverse primer:
AGGCGGGATGAGGAGGCTGG
Product size: 122
CD68
Forward primer:
GGTGGAGGAGAGGCCTGGGG
Reverse primer:
GCTGCGTGGGGGAAGGACAC
Product size: 203 bp

6.12.2 Isolation of whole cell RNA

Isolation of RNA was performed with the RNeasy Mini kit from Qiagen. Cells were infected with the virus containing the desired knockdown vector, and RNA was isolated 48 hours post Puromycin selection. Cells were lysed by adding 350μl buffer RLT directly to the 6-well plate. Cell lysates were then transferred to a 1.5ml eppendorf tube containing 200μl RNAse free H_2O and 350μl Buffer RLT. The lysate was then pipetted directly into a QIAshredder spin column placed in a 2ml collection tube and centrifuged for 2 min at full speed. 900μl of 70% ethanol was added to the homogenised lysate and the mix was transferred to a RNeasy spin column and centrifuged for 15s at 8,000g. The column membrane is washed with 700μl of Buffer RW1 then twice with 500μl Buffer RPE. The RNA was eluted in 14μl RNAse free H_2O. RNA concentration was determined by measuring 2μl on the NanoDrop™ machine.

6.12.3 Eliminating DNA from RNA sample

DNA was removed with the TURBO DNA-free™ kit from Applied Biosystems. First 1.5μl 10x TURBO DNase Buffer and 1μl TURBO DNase were added to the RNA and the sample was incubated at 37°C for 30 minutes. To inactivate the reaction 1.5μl DNase inactivation reagent were added to the solution and incubated for 2 minutes at room temperature. The sludge was then centrifuged at 10,000 g for 1.5 minutes and the supernatant was transferred to a fresh tube.

6.12.4 Reverse Transcription cDNA synthesis

The cDNA synthesis reaction was performed with the RevertAid™ M-MuLV Reverse Transcriptase kit from Fermentas optimised of use in a two-step RT-PCR. A total of 1μl of template RNA was used per reaction and mixed with 0.2μg of random hexamer primers. The reagents were then added in the following order, 4μl 5x reaction buffer, 0.5μl RiboLock™ RNase Inhibitor, 2μl of a 10mM dNTP mix, and finally 1μl of the RevertAID™ M-MuLV Reverse Transcriptase. The total volume of the mix should be 20μl.The RT mix is incubated at 20°C for 10 minutes followed by 30 minutes at 50°C and later filled to 100μl with H_2O. A negative control reaction without the M-MuLV Reverse Transcriptase was performed for every sample.

6.12.5 PCR

The PCR reaction was performed with the iQ™ SYBR® Green supermix. A master mix is made by pipetting 5μl of the BioRad Supermix, 0.25μl of a forward and reverse

primer mix and 3.75μl H_2O per reaction. A total of 1μl of the diluted cDNA is analysed per reaction. All reactions were done in triplicate, except for the negative control (from section 6.12.4). Analysis was done with a BioRad RT machine.

6.13 FACS

The FACS experiment was performed with a phycoerythrin conjugated FGFR3 antibody (PE-FGFR3ab) and a PE-IgG antibody as a negative control. The staining was performed in a staining buffer containing 0.5% BSA 1% FBS and 0.1% NaN_3. U2OS cells were grown to 50% confluency on a 100mm dish. To detach, cells were incubated in 3ml PBS-EDTA at 37°C for 10-15 min. until cells looked rounded up and detached under the light microscope. Cells were mixed thoroughly by pipetting up and down, and a 200μL aliquot was used to count the cells the rest were then spinned down at 500g for 3 min, and then washed three times in the staining buffer. Cells were then diluted in PBS to a concentration of 4000 Cells/μl. 10,000 cells or 25μl were incubated in 10 μl of the respective antibody at 4°°C in the dark for 45 minutes. After the staining cells were then washed two times in staining buffer and finally dissolved in 400μl for measurement.

6.14 Sensitivity of osteosarcoma cells for FGFR3 inhibitors.

Sensitivity of Osteosarcoma was tested with PD173074 (Mw 523.67 g/mol) and Masatinib (Mw 498.65 g/mol) at different concentrations ranging from 0-20,000 nM. Both inhibitors were dissolved in DMSO as indicated by the supplier. The osteosarcoma cell lines tested were U2OS, SaOS and MG63. ≈5,000 cells were plated per well on a 96-well format, and the inhibitors were administered for 72 hours. Cell viability was measured with the Cell-Titre Glo Luminescent Cell Viability Assay kit from Promega. All measurements were performed in triplicate.

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WISSENSCHAFTLICHE BERUFSERFAHRUNG

März 2009-März 2009	**Masterarbeit in Molekulare Genetik und Entwicklungsbiologie**
Projektbeschreibung	Entwicklung eines reversen Genetiks RNAi screening Methode um inhibitoren des p53/pRB Signalwege in menschliche Krebszelllinien zu finden.
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April-Mai 2008	**Praktikum in molekulare Medizin und Genetik**
Projektbeschreibung	Implementation einer multiplexe Drug-Screening Technologie in menschliche Zellkulturen.
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Juli-August 2008	**Praktikum in neuronale Entwicklungsbiologie und Zellbiologie**
Projektbeschreibung	Lokalisierung von 3'-UTR-EGFP Fusionkonstrukte in primäre hippocampale Neuronen von Ratten.
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Juli 2003	**Praktikum in Sandoz Antibiotic Research Laboratories**
Projektbeschreibung	Synthese hochspezialisierte Aminosäuren für die Antibiotiker Forschung.
Unternehmen	Sandoz Vienna
Betreuer	Dr. Klaus Thirring

NICHT-WISSENSCHAFTLICHE BERUFSERFAHRUNG

September-Oktober 2008	Radio Reportage: Nepal
Projektbeschreibung	Dreiwochige Aufenthalt in Nepal um unter anderem eine 20min Reportage über die neulich gewählte Maoistische Regierung zu produzieren.
Unternehmen	FM4 Reality Check. ORF Radio. http://fm4.orf.at/radio/stories/fm4realitycheck
September-Oktober 2008	Radio Reportage: Sri Lanka
Projektbeschreibung	Zweiwochige Aufenthalt in Nepal um unter anderem eine 20min Reportage über den Konflikt zwichen Tamilen und Hindi zu produzieren.
Unternehmen	FM4 Reality Check. ORF Radio. http://fm4.orf.at/radio/stories/fm4realitycheck
March-April 2007	Radio Reportage: Kenya
Projektbeschreibung	Sechswöchige Aufenthalt in Kenya um unter anderem eine 20min Reportage über die Rolle der katholischen Kirche im Kampf gegen die Armut in Kenya
Unternehmen	OE1 Religion. ORF Radio. http://oe1.orf.at/programm/
Mai 2005-Gegenwart	Barkeeper, Kellner
Unternehmen	Manolos Bar Restaurant. http://www.manolos.at/

PRAKTISCHE ÜBUNGEN AN DER UNIVERSITÄT

Cyto- und Embryogenetik in Medaka Fisch, Komputergraphik und molekulare Modellierung, Bioinformatikübung, Biochemie, Molekulare Biologie I-III, Analytische Chemie, Organische Chemie, Strukturbiologie I-II, Grundlagen der Chemie

IKT Kentnisse

Informatikkentnisse	Microsoft Office; LaTeX; Windows; MacOS; Linux; Internet; Relevante Molekularbiologische Datenbanken (Ensembl, PDB, ZFIN, BLAST, Phylogen, Rfam etc…); NetLogo ,Vienna RNA package, whatif, charmmed, Prism, Adobe Photoshop

SPRACHENKENNTNISSE

Muttersprache(n)	**Spanisch, Englisch**
Andere Sprache(n)	**Deutsch (Ausgezeichnet), Französisch (Mäßig)**

PERSÖNLICHE STÄRKEN

Lernbereitschaft und Flexibilität
Ausdauer und Belastbarkeit
Selbstständigkeit und Zuverlässigkeit
Anpassungsvermögen und Multikulturelles Hintergrund