3 A TRAIL APOPTOSIS ASSAY AND COMPARISON OF RNAI SCREENING METHODS

There are many ways in which an RNAi screen can be conducted. RNAi can be induced using chemically synthesised RNA oligonucleotides (siRNAs) or using RNA hairpins (shRNAs) expressed from plasmid vectors. Screens can also be performed using a gene-by-

gene strategy, where the screen is performed in a microtitre plate with each well containing a construct or constructs targeting one gene, or using a pooled strategy, where constructs targeting many genes are introduced into a single population and some form of selection applied, followed by the identification of hairpins that are enriched, after this selection.

This chapter presents firstly, the establishment of an assay for a process of biological and medical interest that can be altered using RNAi, namely an assay for TRAIL-induced apoptosis (see section 1.3). This assay is optimised to provide the greatest differentiation between negative and positive controls for genes whose knock-down alters the function of the pathway.

Secondly, this assay is used to compare two different methods for inducing RNAi – namely transfection of siRNAs and transfection of vectors encoding shRNAs.

3.1 Introduction

3.1.1 Assays for apoptosis

In order to screen for genes involved in TRAIL mediated apoptosis, a method for measuring the apoptosis caused by treatment with TRAIL is required. There are many methods for the detection of apoptosis. These can be divided into two strategies. The first is to utilise methods that measure events that are specifically associated with apoptotic cell death (reviewed in (Huerta et al. 2007). These include: the examination of cell morphology by electron or fluorescent microscopy, methods for the detection of DNA fragmentation, Annexin V staining, measurement of the activity of apoptosis effector proteins, such as caspases, and the detection of the cleavage targets of caspases. While these methods provide an accurate and reliable readout of apoptosis, they are often time consuming and therefore unsuitable to high-throughput screening applications. Those that can be adopted for a high-throughput screen are generally prohibitively expensive for the academic laboratory.

The second strategy is to measure the loss in cell viability caused by treatment with a known apoptosis inducing agent. While this does not specifically measure the level of apoptosis, interesting results can be followed up using a lower throughput method that specifically measures apoptosis to confirm that this is the cause of the loss of cell viability.

There are several methods for measuring cell viability. The simplest are dye exclusion assays. In these assays cells are stained with dyes such as Trypan Blue or Propidium Iodide, which are excluded from living cells, but readily stain cells with compromised membrane integrity. The number of stained and unstained cells can be counted using microscopy. While

simple, these assays are also time consuming due to the necessity for microscopy. There are several dyes that measure viability by measuring metabolic activity of cells. One such dye is alamarBlue. In its native, oxidised form alamarBlue is a blue, non-fluorescent compound. When reduced by the action of respiring cells on the culture media, the dye becomes red and fluorescent, as measured by excitation at 360-530 nm and monitoring emission at 590nm. alamarBlue requires no processing of samples, is non-toxic to both the cells being studied and the user, and is cheap. An alamarBlue assay for the effect of TRAIL on the viability of HeLa cells was successfully used by Aza-blanc *et al* in a previous screen for regulators of the TRAIL-induced apoptosis pathway (Aza-Blanc et al. 2003). The assay involved transfecting cells in duplicate with the RNAi inducing agent, and then treating one duplicate with the TRAIL ligand and the other with media alone. After 24 hours the viability of the cells in both replicates is measured by incubating the cells for 4 hours in media with 10% alamarBlue and measuring the fluorescent emission from the wells (Figure 3.1**a**). The effect of the TRAIL on the cells is expressed as the percentage of cells that survive using the following equation:

$$survivial = \frac{\bar{fl}_{treated}}{\bar{fl}_{untreated}} \times 100\%$$

Equation 1

where fl_x is the fluorescent signal of well type x and $\bar{fl}_x$ is the mean of all wells of type x. Where a particular experiment contains several treated and several untreated wells, the average survival is the ratio of two means – the ratio of the mean fluorescence in treated wells to the mean fluorescence in untreated wells. Since the average survival is a ratio of two means the standard deviation of this value cannot be directly calculated, but is estimated thus:

$$\sigma_{survival} = survival \sqrt{\left(\frac{\sigma_{fl_{untreated}}}{\bar{fl}_{untreated}}\right)^2 + \left(\frac{\sigma_{fl_{treated}}}{\bar{fl}_{treated}}\right)^2}$$

Equation 2

Where σ_{fl_x} is the standard deviation of the fluorescent signal from wells of type x. This makes the application of statistical methods such as Student t's tests more complex. This only applies in situations where an individual experiment contains multiple replicate wells. For example this does not apply if an experiment is repeated several times, but each individual experiment contains only one treated and one untreated well, particularly if they are both on the same plate. Here the survival in each experiment is simply the ratio of the individual values in each replicate and the mean survival is the mean of the survival values

for each replicate.

Here the survival is a measurement of the change in cell viability rather than a direct measurement of the induction of apoptosis. As such it strictly measures TRAIL-induced cytotoxicity rather than TRAIL-induced apoptosis and the ability to specifically induce apoptosis of any new gene isolated using this assay must be tested.

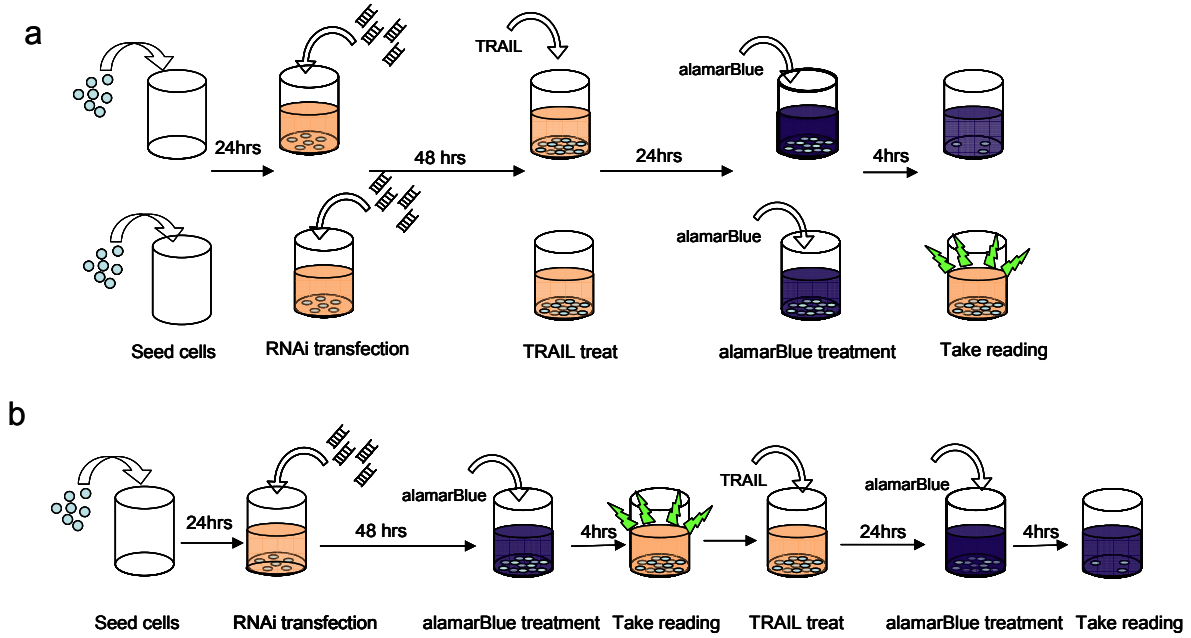

Figure 3.1 TRAIL cytotoxicity assays.
a) Treated vs. untreated assay as used by (Aza-Blanc et al. 2003). Schematic representation of assay. Cells are seeded and transfected in duplicate. After 48 hours one of the two duplicates is treated with TRAIL ligand for 24 hours. The viability of both duplicates is then assessed using alamarBlue. See Equation 1 for calculation of average survival and Equation 2 for estimation of standard deviation. b) Before vs after protocol. Schematic representation of assay. Cells are seeded and transfected. After 48 hours viability is assessed using alamarBlue, followed by treatment with the ligand. Viability is assessed again after 24 hours. See Equation 6 for calculation of survival

3.1.2 The pSHAG-MAGIC vector and the Expression Arrest library

Hairpin encoding vectors in this chapter come from, or are constructed using the same principles as the Expression Arrest Library (v1.3)(Silva et al. 2005) which was kindly made available by Prof. G. Hannon. Constructs in this library are designed to mimic the structure of natural miRNA precursors and are termed shRNA^{mir}s. The constructs were designed by remodelling the human miRNA miR-30 to include a sequence targeting the gene of interest. This remodelled miRNA is inserted into a vector containing 125bp of 5' and 3' sequence from the primary miR-30 transcript. Thus, when transcribed the sense and antisense hairpin structures are flanked by miR-30 leader and termination sequences and linked by the miR-30 hairpin loop. It has been reported that hairpins designed thus are up to 12 times more efficient than standard shRNAs (Silva et al. 2005).

The shRNA^{mir} constructs are cloned into the shRNA^{mir} expression vector pSHAG-

MAGIC2 (pSM2). The vector is based on a self-inactivating Mouse Stem Cell Virus (MSCV). The hairpin is cloned between the viral Long Terminal Repeats (LTR). Hairpins are expressed from the U6 promoter. The vector includes sequences for three selectable markers: kanamycin, chloramphenicol and puromycin. The kanamycin selectable marker is outside the viral LTRs, while the chloramphenicol (for selection in bacterial cells) and puromycin (for selection in mammalian cells) markers are between the viral LTRs. The vector also contains a molecular barcode that allows identification of the vector from within pools. Unfortunately, at the time of writing, the sequences of the barcodes are not available.

Release 1.3 of the Expression Arrest library contains 18,882 bacterial clones containing vectors encoding hairpins targeting 13,456 unique predicted or confirmed Refseq transcripts. The library is provided arrayed in 96 well plates of glycerol stocks with each well containing one clone. The sequence of the hairpins was confirmed before the library was released. In addition 960 clones were sequenced when the library was obtained; 88% of clones contained the expected sequence.

3.1.3 Measurements of the effectiveness of a screen

In order to develop a high-quality assay and compare different screen methods it is necessary to have a metric to assess the effectiveness of a screen. The obvious statistics for assessing the quality of a screen are the sensitivity and accuracy (otherwise known as positive predictive value and related to the false positive rate), defined as follows:

$$sensitivity = \frac{TP}{TP + FN} x100\%$$

Equation 3

$$accuracy = \frac{TP}{TP + FP}$$

Equation 4

where TP is true positives, FN is false negatives and FP is false positives. While this information tells us the ultimate success of a screen, it relies on knowing where the true "hits" are, and gives little information on the magnitude of the differences between the true positives and true negatives. Two statistical measures that address these flaws are the signal to noise (S/N) and signal to background (S/B) ratios. They measure the size of the difference between the positive and negative controls. However, they incorporate either no information on variation (S/B), or information on the variation of only one of the values (S/N). Zhang *et al* proposed a new measure of separation between two populations (e.g.

positive and negative values) named the Z'-factor, which is defined as the ratio of the "signal band" to the dynamic range (Zhang, Chung & Oldenburg 1999):

$$Z' = 1 - 3\frac{\sigma_+ + \sigma_-}{|\mu_+ - \mu_-|}$$

Equation 5

where μ_+,σ_+, μ_- and σ_- are the mean and standard deviation of the positive and negative controls, respectively. The value of Z' ranges from 1 (the perfect assay) to -∞. Zhang *et al* propose using the following categories to interpret the Z'-factor:

Z'-Factor	*Meaning*
1	The perfect assay
0.5-1	An excellent assay which could provide quantitative information
0-0.5	A usable assay that may provide limited quantitative information
0	A Yes/No Assay
<0	Screen essentially impossible

Table 3-3 Interpretation of the Z-factor values

Strictly Standardised Mean Difference (SSMD) has been proposed as an alternative to the Z-factor (Zhang 2007). The main advantage of SSMD over Z-factor is that it has a clear probability interpretation. However, this calculation is only trivial when the data considered is normally distributed. Since the data dealt with here are ratios, and therefore not normally distributed, there would seem to be no advantage in using SSMD over the more widely recognised Z'-factor.

3.2 Demonstration of cytotoxicity of TRAIL

In order to demonstrate the sensitivity of HeLa S3 cells to the TRAIL ligand, and to set up an assay that could be used to compare methods for screening RNAi libraries, the assay used in (Aza-Blanc et al. 2003) was adapted. When the assay was directly scaled for a 96-well system the cells showed little or no sensitivity to the TRAIL ligand (see Figure 3.2a). A series of optimisation experiments was undertaken to improve the sensitivity of HeLa S3 cells to the TRAIL ligand. Examples of representative results from this process are presented in Figure 3.2. Factors investigated include the type of plate used (standard tissue culture plate vs. Falcon OptiLux™ white walled tissue culture plate, data not shown), concentration of serum in the assay media (Figure 3.2b), length of treatment (Figure 3.2c,d), the protocol used for seeding the cells (data not shown), number of cells seeded into each well (Figure 3.2d) and concentration of the ligand (Figure 3.2d). It was found that using white-walled plates eliminated fluorescent cross-talk between wells in the plate. Cytotoxicity was improved by a

new seeding protocol which allowed cells to adhere to the surface of the plate before the plate was placed in a moist box and placed in the 37°C, 5% CO_2 incubator. Cytotoxicity was also improved using a lower concentration of serum in the assay medium and using a smaller number of cells (Figure 3.2b and Figure 3.2d). Neither the concentration of the ligand nor the length of treatment seemed to have a large effect on the sensitivity of the cells to TRAIL, except at very low cell densities where treating for 48 hours rather than 24 slightly increased the sensitivity (Figure 3.2c,d). Using 2,500 cells and treating with 1.26μg/ml TRAIL for 24 hours in serum-free media led to a survival of only 31%, and treating for 48 hours led to a decrease in survival to only 13% when compared to untreated cells

These figures compare to an average survival of 38.5% reported previously (Aza-Blanc et al. 2003), where a higher density of cells were used, and cells were treated in medium with 1% serum, and demonstrate that HeLa S3 cells are sensitive enough to allow high-throughput screening for genes that reduce sensitivity to TRAIL ligand.

3.3 Rescue of TRAIL induced cytotoxicity

3.3.1 Rescue of TRAIL induced cytotoxicity by siRNA

Caspase 8 is a key gene in the TRAIL-induced apoptosis pathway (see section 1.3.1). In order to demonstrate that TRAIL-induced cytotoxicity can be modified by RNAi, siRNAs targeting either the Caspase-8 gene (siCasp8) or the Luciferase gene (siGL2), as a negative control, were transfected into various numbers of HeLa S3 cells in varying quantities and the cells were then assayed for TRAIL sensitivity. As discussed above, it was found that lower density cells were more sensitive to the TRAIL ligand than higher cell densities. However, protocols for transfection of siRNAs into cells all specify a higher density of cells than those found to be optimal for the highest sensitivity to the TRAIL ligand. As a compromise cell densities of 10,000 and 5,000 cells per well were tested. It was found that cells transfected with siCasp8 became insensitive to TRAIL-induced cytotoxicity, with greater than 80% of cells surviving 24 hour treatment with TRAIL ligand (Figure 3.3). This was irrespective of the quantity of siRNA transfected into the cells, with the exception of cells at a density of 5,000 cells per well transfected with 10pmol of siCasp8 (Figure 3.3). However, given the large error in that measurement, and that cells at a density of 10,000 cells per well transfected with the same amount of siRNA were resistant to TRAIL-induced cytotoxicity, it seems likely that this datum point is an anomaly possibly due to a failure of transfection. In contrast, cells transfected with siGL2 were sensitive to TRAIL-induced cytotoxicity

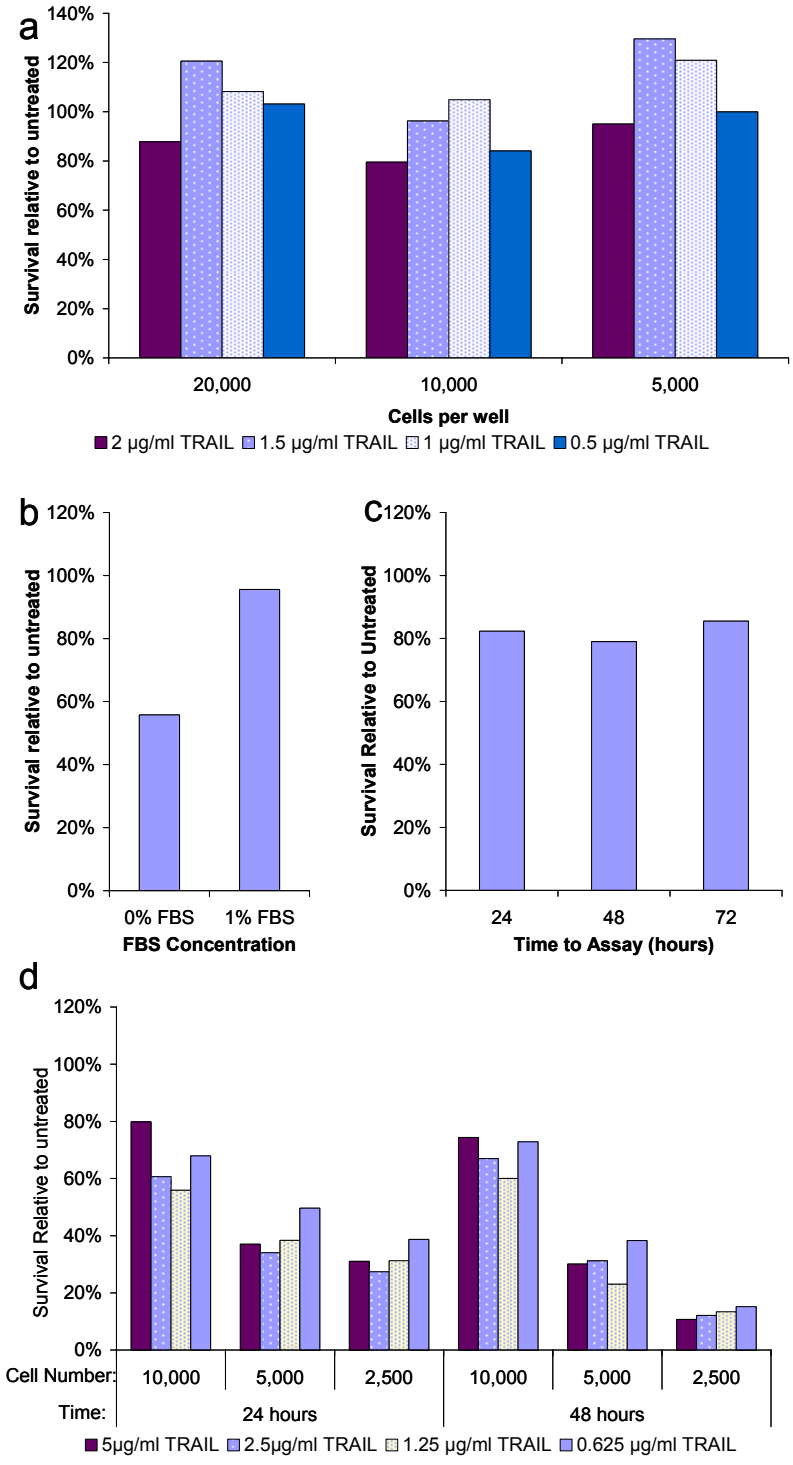

Figure 3.2 HeLa S3 cells are sensitive to TRAIL
a) A direct adaptation of the assay used in by Aza Blanc *et al.* (Aza-Blanc et al. 2003)to a 96 well system. Minimal cytotoxicity was observed under all conditions tried. b) Effect of serum concentration sensitivity to TRAIL. 10,000 cells were seeded and grown for 60 hours and treated with either 1µg/ml TRAIL or media with concentration of serum indicated for 24 hours and viability assessed using alamarBlue. c) Effect of length of treatment on sensitivity to TRAIL. 10,000 cells were seeded and grown for 60 hours and treated with either 1µg/ml TRAIL in serum-free media or serum-free media for the length of time indicated and viability assessed using alamarBlue. d) Effect of cell number and TRAIL concentration on sensitivity to TRAIL. Number of cells indicated were seeded and grown for 60 hours and treated with the concentration of TRAIL indicated in serum-free media or serum-free media alone for the length of time indicated and viability was assessed using alamarBlue. All data are means of two replicates.

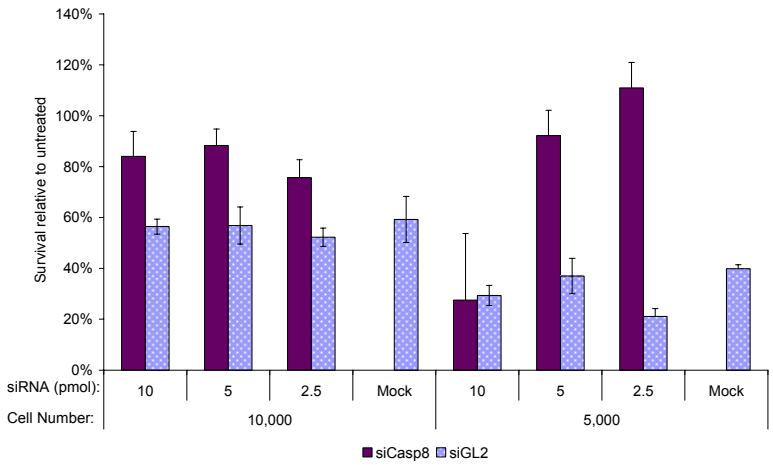

Figure 3.3 siRNA mediated knock-down of Caspase-8 rescues TRAIL-induced cytotoxicity
The number of HeLa S3 cells indicated were seeded and grown for 24 hours. Cells were then transfected with the indicated amount of either siCasp8 or siGL2. After 48 hours cells were treated with either 1μg/ml TRAIL in serum-free media or serum-free media alone for 24 hours and viability assessed using alamarBlue. Data are means of three replicates. Error bars represent one standard deviation.

(Figure 3.3). While the density of cells had little effect on the sensitivity of cells transfected with siCasp8, negative control transfected cells at a density of 5,000 cells per well, were more sensitive to TRAIL-induced cytotoxicity than those at a density of 10,000. All cells seeded at a density of 5,000 cells per well and transfected with 2.5pmol of siCasp8 survived 24 hours of treatment with TRAIL, compared to 21% of cells seeded at the same density and transfected with the same quantity of siGL2. These results demonstrate that RNAi targeted against TRAIL pathway members can disable the pathway and rescue cells from TRAIL-induced cytotoxicity.

3.3.2 Construction of pSM2.shCasp8.1/2

In order to test the effectiveness of plasmid based expression of shRNA^{mir}s in modulating TRAIL-induced cytotoxicity, plasmid vectors containing sequences encoding hairpins targeting the Caspase 8 gene were constructed in the vector pSHAG-MAGIC-2c (pSM2). The sequence used to synthesise siCasp8 is a 21bp sequence, whereas the sequences used to generate shRNA^{mir} insets are 22bp sequences and have mismatches at the 3' end of the guide strand. Since simply extending the siCasp8 sequence by a base in the 5' or 3' direction may result in a suboptimal hairpin sequence, three new sequences targeting Caspase 8 were designed using shRNA retriever tool (http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA). These sequences were synthesised as 97bp oligonucleotides including miR30 leader, termination and loop sequences. These oligonucleotides were PCR amplified using oligonucleotides containing miR30 leader/termination sequences and either an EcoRI or an XhoI restriction

site. The PCR product was A-tailed and cloned into the holding vector pGEM-T using TA cloning. Several colonies containing the pGEM-hairpin constructs were sequenced to verify the sequence of the hairpin. Hairpins containing the correct sequence were sub-cloned into pSM2 by digesting the pGEM-hairpin construct with EcoRI/XhoI, purifying the fragment of the correct size from an agarose gel and ligating into pSM2 vector digested with the same enzymes. The final constructs were verified by sequencing. In this manner two vectors were successfully constructed which expressed two different hairpins targeting Caspase 8 – pSM2.shCasp8.1 and pSM2.shCasp8.2 (see 2.5.3).

3.3.3 Optimisation of DNA transfection

In order to use plasmid-based shRNA^{mir}-mediated gene silencing to modulate TRAIL-induced apoptosis, it is necessary to introduce the plasmids into the cells being studied at a high efficiency. To test the efficacy of a range of lipid-based transfection reagents, HeLa S3 cells were transfected with plasmid expressing an enhanced GFP protein (pEGFP), allowing for the visualisation of transfected cells. The reagents Lipofectamine 2000 and Effectene gave appreciable transfection efficiency of 36% and 38%, respectively, as calculated by the ratio of the number of green fluorescent cells (number of cells transfected) to the number of DAPI-stained nuclei (total cell number). However, these reagents were associated with very high toxicities, as calculated by the ratio of DAPI-stained nuclei in wells transfected with pEGFP to the number of DAPI-stained nuclei in untransfected wells. In total this gave a very small total number of transfected cells. In contrast, cells transfected with either GeneJuice or siPort XP-1 showed much lower toxicities, but little or no transfection, again resulting in a very small total number of transfected cells (**Table 3-4**).

	HeLa S3		*HeLa*	
	Transfection Efficiency	*Viability*	*Transfection Efficiency*	*Viability*
Lipofectamine 2000	36.80%	2.90%	37.50%	3.40%
Effectene	48.50%	7.90%	12.86%	3.40%
GeneJuice	0.50%	43.10%	8.90%	55%
siPort XP-1	1.34%	56%	12.20%	61.20%

Table 3-4 Comparison of transfection reagents.
HeLa S3 cells or HeLa cells were seeded on an 8 well slide and grown for 24 hours. Cells were then transfected with pEGFP-N1 plasmid using the indicated transfection reagent. Cells were fixed and stained with DAPI after 48 hours. Transfection efficiency was calculated as the ratio of fluorescence cells to DAPI-stained nuclei. Viability was calculated as the ratio of DAPI-stained nuclei in transfected wells to DAPI-stained nuclei in untransfected wells.

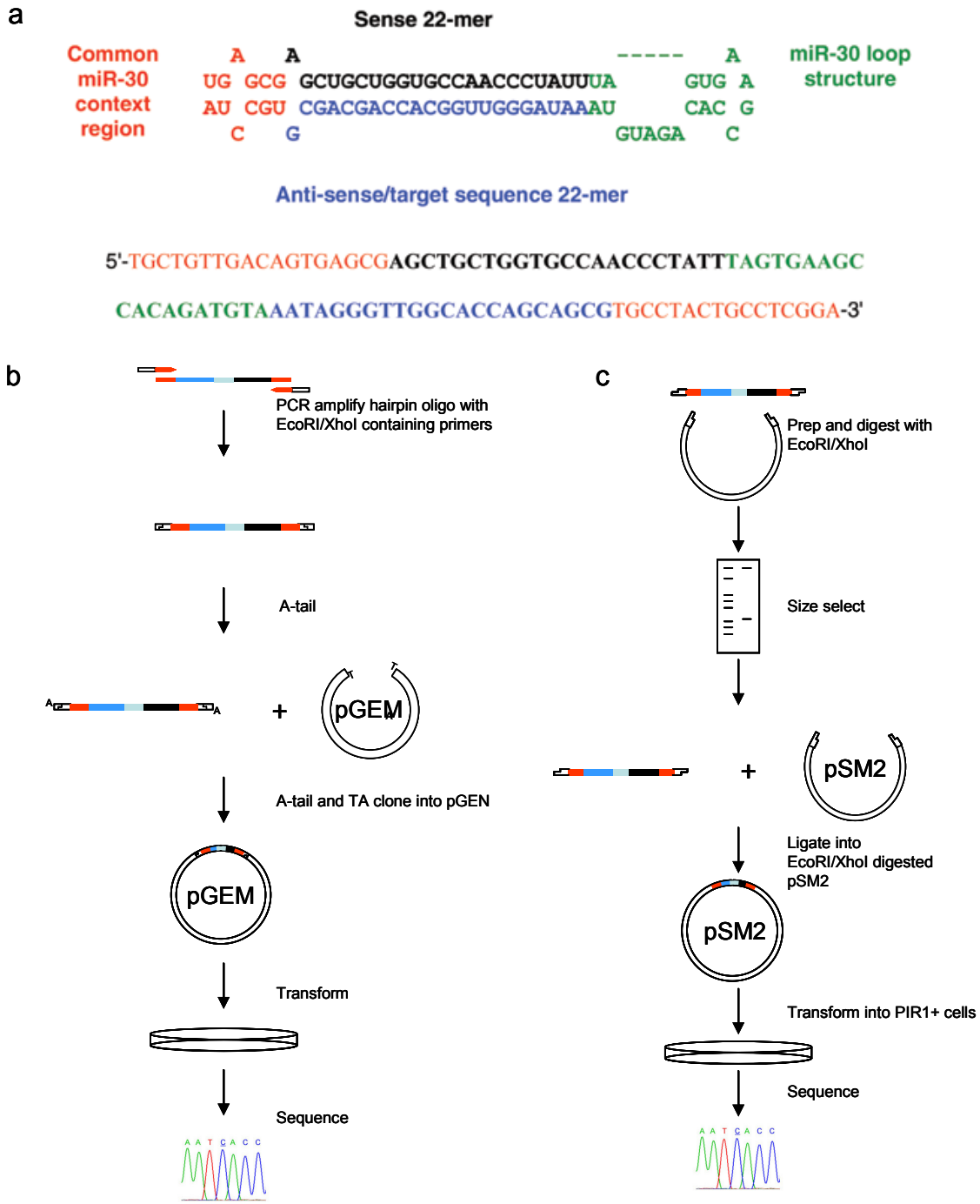

Figure 3.4 Cloning of shRNA^{mir} constructs in the pSM2 vector
a) Above, the structure of the hairpin RNA. Below, the structure of the DNA oligonucleotide template used to generate the constructs. Colours represent the origin of the sequence: Red - miR-30 flanking sequence. Green – miR-30 loop sequence. Blue: sense and anti-sense target sequence respectively. Taken from (Paddison et al. 2004a). b) Cloning of hairpin insert into pGEM holding vector, see text and methods for details. c) Subcloning of hairpin insert into pSM2 by restriction digest. See text and methods for details.

In order to determine if better transfection efficiency could be achieved, the experiment was repeated using HeLa cells. HeLa cells are the parent cell line of HeLa S3 cells and are larger, flatter and adhere to the growth surface more tightly. Transfections using Lipofectamine 2000 and Effectene gave similar results in HeLa cells to those obtained in

HeLa S3 cells. However, transfection using both GeneJuice and siPort XP-1 gave transfection efficiencies an order of magnitude greater than those observed in HeLa S3 cells, while still showing the same low toxicity (**Table 3-4**). To determine if HeLa cells could be used in the TRAIL assay instead of HeLa S3 cells, the sensitivity of HeLa cells was assessed. At higher cell densities HeLa cells seemed insensitive to TRAIL-induced cytotoxicity. However, at very low densities similar numbers of cells survived TRAIL treatment to those seen for 5,000 HeLa S3 cells. Again, both concentration of TRAIL and length of treatment had minimal effect on the sensitivity of the cells (Figure 3.6)

The pSM2 plasmid contains a puromycin resistance marker. Reasoning that selecting for transfected cells after transfection might increase the transfection efficiency by reducing the number of untransfected cells, cells were co-transfected with pEGFP-N1 and pSM2 and then treated with either puromycin for 24 hours to select transfected cells, or grown in media alone for the same length of time.

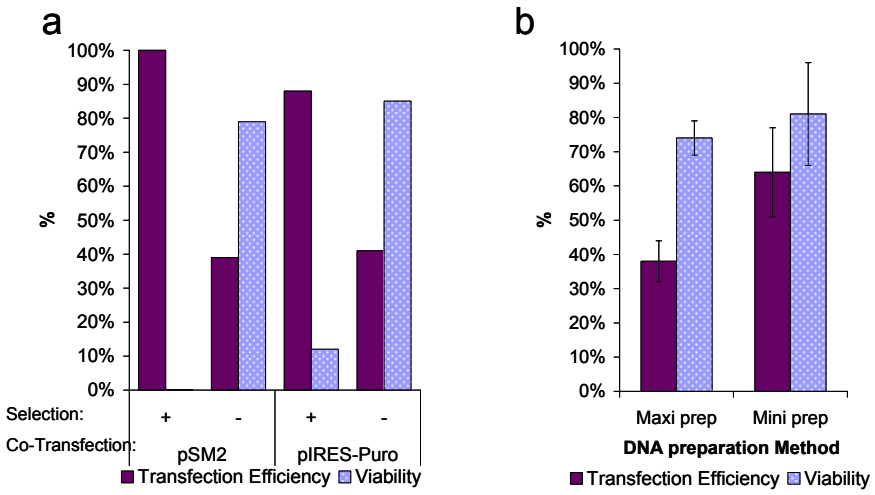

Figure 3.5 Selection of transfected cells using puromycin.
a) Effect of drug selection on transfection efficiency. HeLa cells were seeded on 8 well slides and grown for 24 hours. Cells were co-transfected with either pEGFP-N1 and pSM2 or pEGFP-N1 and pIRES-P. After 24 hours cells were treated with 2μg/ml puromycin. Slides were fixed and stained after a further 24 hours. b) Effect of DNA preparation method on transfection efficiency. HeLa cells were seeded on 8 well slides and grown for 24 hours. Cells were then transfected with pEGFP-N1 prepared using Qiagen Plasmid Mini kit or Qiagen Endotoxin-free HiSpeed Maxi kit. Slides were fixed and stained with DAPI 48 hours later. Data are means of three replicates; error bars represent one standard deviation. Transfection efficiency was calculated as the ratio of fluorescence cells to DAPI-stained nuclei. Viability was calculated as the ratio of DAPI-stained nuclei in transfected wells to DAPI-stained nuclei in untransfected wells.

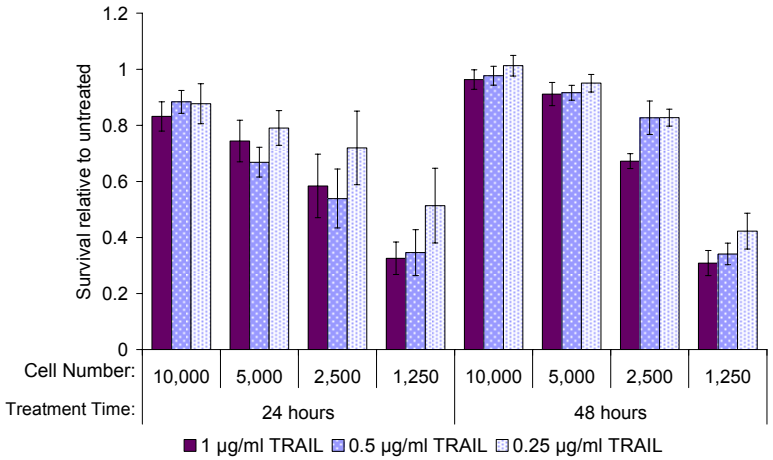

Figure 3.6 HeLa cells are sensitive to TRAIL.
The numbers of HeLa cells indicated were grown for 72 hours. Cells were then treated with either the concentration of TRAIL as indicated, in serum-free media or serum-free media alone for the length of time indicated and viability was assessed using alamarBlue. Data are means of three replicates. Error bars represent 1 standard deviation

Where cells were not selected, transfection efficiencies of approximately 40% were observed. Selection for pSM2 transfected cells using puromycin gave near zero surviving cells. In contrast, 12% of cells co-transfected with pEGFP-N1 and pIRES-p (another plasmid encoding a puromycin resistance marker) were resistant to puromycin selection. Furthermore, these surviving cells were significantly enriched in transfected cells (88% for selected cells compared with 41% for unselected cells, **Figure 3.5**c). These results demonstrate that selecting puromycin resistant cells can increase transfection efficiency when a vector with functional puromycin resistance was used. However, it seems that for unexplained reasons this is not the case for pSM2. One difference between the pIRES-P plasmid and the pSM2 plasmid was the method used to prepare the DNA. The pIRES-p plasmid was prepared using an endotoxin-free HiSpeed maxi prep kit from Qiagen, while pSM2 was prepared using a plasmid mini prep kit from Qiagen. To investigate if this could be the source of the differences seen, pEGFP-N1 plasmid DNA prepared with either an endotoxin-free HiSpeed maxi prep kit or a plasmid mini prep kit was transfected in to HeLa cells and the transfection efficiency assessed. The method of DNA preparation had no significant effect on the efficiency of the transfection ($p = 0.41$, **Figure 3.5**d).

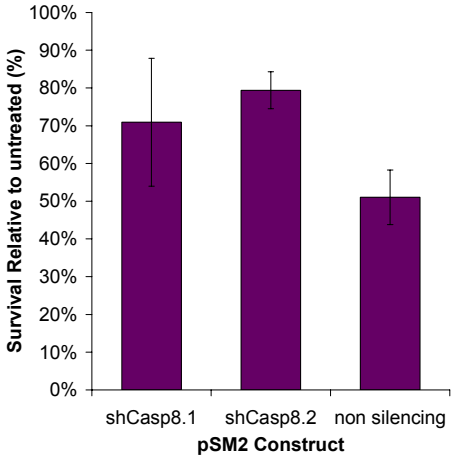

Figure 3.7 shRNAmir mediated knock-down of Caspase-8 rescues TRAIL-induced cytotoxicity
2,500 HeLa cells were seeded and grown for 24 hours. Cell were then transfected with pSM2.shCasp8.1, pSM2.shCasp8.2 or pSM2.shControl (a hairpin targeting a pseudo-gene) using siPort XP-1. After 48 hours cells were treated with either 1μg/ml TRAIL in serum-free media or serum-free media for 24 hours. Viability was assessed using alamarBlue. Data are mean of three replicates. Error bars represent 1 standard deviation.

In order to demonstrate that TRAIL-induced cytotoxicity can be modulated by shRNA^{mir} mediated RNAi, HeLa cells were transfected with pSM2 constructs targeting either the Caspase 8 gene (see 0) or a pseudogene (shControl), and the sensitivity of cells to TRAIL ligand was assessed. 51% of cells transfected with the control construct survived 24 hours treatment with TRAIL ligand, while 71% and 79% of cells survived when transfected with shCasp8.1- and shCasp8.2-containing constructs (Figure 3.7) — a 1.4-fold and 1.54-fold relative increase in survival, respectively

The assay used by (Aza-Blanc et al. 2003) and above compares the average viability of treated cells to that of untreated (Figure 3.1**a**). This method of calculating survival has several drawbacks. Firstly, since the survival is expressed as a ratio of averages, the standard deviation must be estimated from the standard deviations of the average viability of treated and untreated wells. Secondly, all transfections must be carried out in duplicate, using twice the amount of siRNA, transfection reagent, assay reagent etc. Thirdly, while comparing treated to untreated wells takes account of the effect of the siRNA on the basal viability of the cells, it does not take account of variation in the seeding of cells between wells. An alteration of the assay to compare viability before and after treatment in the same well addresses these issues. Here cells are seeded and grown for 24 hours before being transfected. After another 48hours their viability is assessed and they are treated with TRAIL. The viability is reassessed 24 hours later (Figure 3.1**b**). Here the survival is calculated per well, using:

$$survival = \frac{fl_{after}}{fl_{before}} x100\%$$

Equation 6

and the average survival is simply the mean of the survival in each well and the standard deviation of survival is the standard deviation of survival in each well.

This new assay protocol was used to study the effect of cell density and the ratio of DNA to transfection reagent on both transfection and assay efficiency simultaneously (Figure 3.2). Increasing the density of cells in both siRNA- and shRNA^{mir}-mediated experiments increased the survival of cells transfected with both control constructs and constructs targeting Caspase 8 or BID, another gene in the TRAIL apoptosis pathway (Figure 3.9a and Figure 3.8b). At 3000 cells per well 38% of cells transfected with pSM2.shControl and 23% of cells transfected with siGL2 survived, while 76% of cells transfected with pSM2.shCasp8.2 and 84% of cells transfected with siCasp8 survived. Transfection of an siRNA targeting BID had a similar but slightly less powerful effect, with 66% of cells surviving, while transfection with pSM2.shCasp8.1 had little effect with 44% of cells surviving.

It is to be noted that a higher percentage of untransfected cells survived than cells transfected with any siRNA or pSM2 construct (Figure 3.8a and Figure 3.8b). However, the same is also true of cells transfected with pEGFP-N1. This suggests that the effect is not due to the engagement of the RNAi machinery, but to the process of transfection itself. It is possible that here toxicity associated with the transfection is affecting the assay (Table 3-4) suggesting where there is no transfection, the cell number is higher than in transfected wells at the time of treatment. However, since these non-specific effects seem to be related to the transfection rather than the construct transfected, the effects should be similar, independent of the siRNA/shRNA^{mir} transfected. Therefore it can be assumed that the differences between different siRNAs/shRNA^{mir}s are the result of the differing effects of that construct on the pathway. This underlines the importance of comparing the effect of an siRNA/shRNA^{mir} to a negative control siRNA/shRNA^{mir} rather than an untransfected sample. The effect of the amount of siRNA transfected on assay outcome has already been investigated. above (Figure 3.3). Using both greater quantities of DNA and greater quantities of transfection reagent increased the difference in TRAIL sensitivity between control transfected cells and cells transfected with pSM2.Casp8.2. A total of 15% of cells transfected with 80ng of pSM2.shControl, using 16 μl of siPort XP-1, survived 24 hours treatment with 1μg/ml TRAIL, while 36% of cells transfected in the same way with pSM2.shCasp8.2

survived. — a 2.4-fold relative increase in survival.

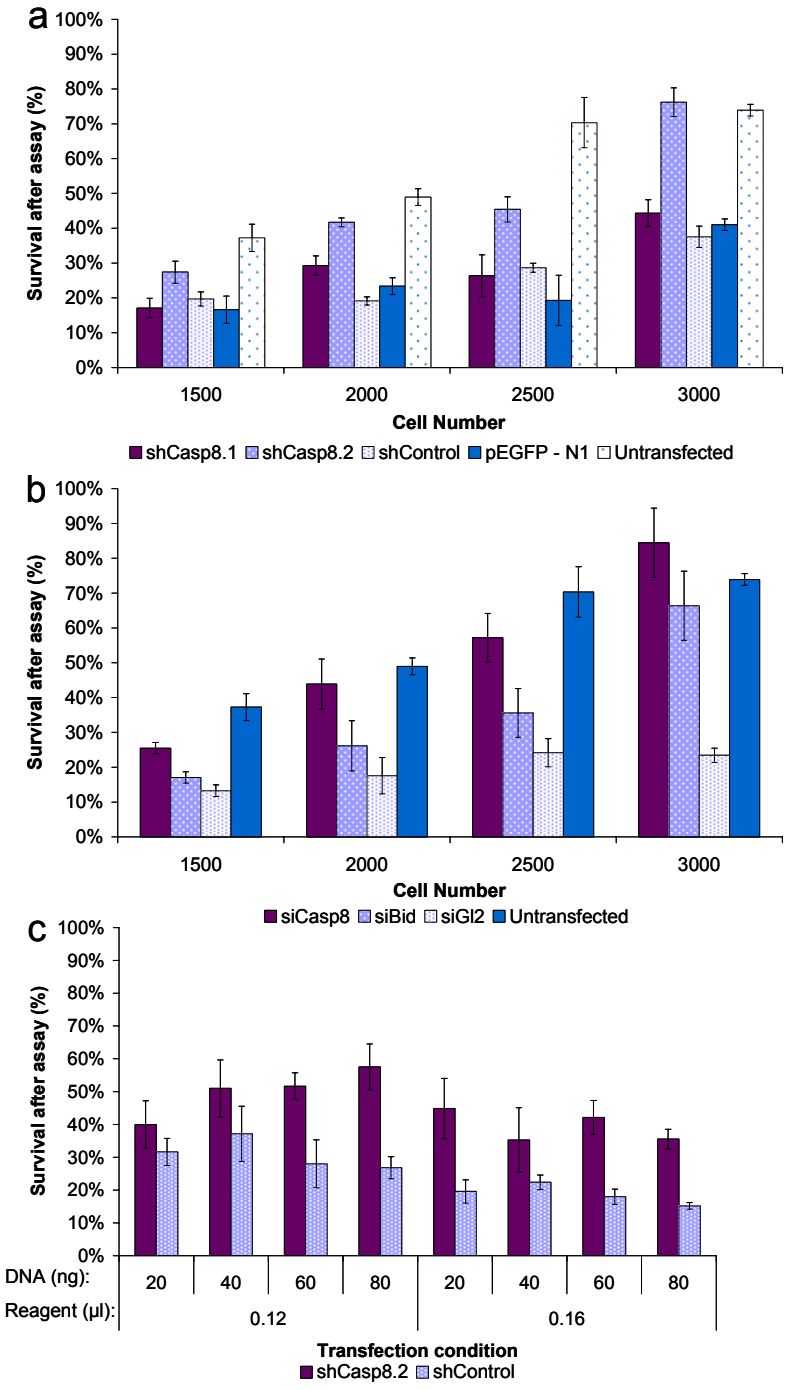

Figure 3.8 Optimisation of RNAi-mediated rescue from TRAIL-induced cytotoxicity .
a),b) Effect of cell number on rescue from TRAIL-induced cytotoxicity. The number of cells indicated were seeded and grown for 24 hours. Cells were transfected with either a) shRNA^{mir} or b) siRNA. After 48 hours cells pre-treatment viability was assessed and cells treated with 1µg/ml TRAIL. Post-treatment viability was assessed 24 hours later. c) Effect of DNA to reagent ratio on shRNA-mediated rescue from TRAIL-induced cytotoxicity. 3000 cells were seeded and grown for 24 hours. Cells were then transfected with the indicated shRNA using amounts of plasmid DNA and transfection reagent as indicated. Cells were grown for a further 48 hours and pre-treatment viability assessed. Cells were then treated with 1µg/ml TRAIL for 24 hours and post-treatment viability was assessed. Error bars represent 1 standard deviation.

Reverse transfection protocols — where cells are overlaid on previously prepared DNA/lipid complexes — have the advantage of saving 24 hours on the traditional transfection process, where DNA/lipid complexes are added to cultures of cells seeded 24 hours earlier. To investigate the feasibility of using this technique here, cells were transfected with pSM2.Casp8.2 or pSM2.shControl under a large range of conditions and the sensitivity to TRAIL was assessed 48 hours later. On average 27% of cells seeded at a density of 4,000 cells per well transfected with 80ng of pSM2.shControl, using 0.24µl of siPort XP-1, survived 24 hours treatment with 1µg/ml TRAIL, while 56% of cells seeded and transfected in the same way with pSM2.shCasp8.2 survived TRAIL treatment (Figure 3.9) — a 2.07-fold relative increase in survival. This suggests that the reverse transfection protocol is nearly as efficient as the traditional protocol, which require an additional 24 hours.

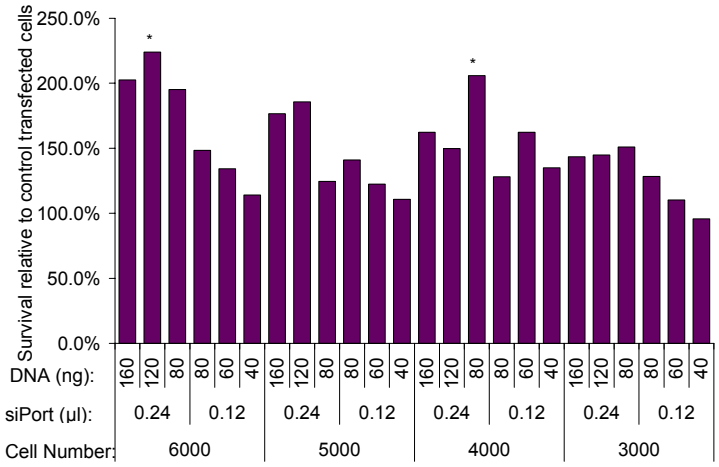

Figure 3.9 Optimisation of RNAi-mediated rescue from TRAIL-induced cytotoxicity using reverse transfection
Transfection complexes were prepared using the amount of DNA and reagent (siPort XP-1) indicated and arrayed in a microtitre plate. The number of cells indicated were seeded on top of the transfection complexes and grown for 48 hours. Pre-treatment viability was assessed and cells were treated with 1µg/ml TRAIL. Post-treatment viability was assessed after 24 hours. Results are expressed as the ratio of survival in pSM2.Casp8.2 transfected wells to the survival in pSM2.shControl transfected wells. * marks the conditions giving the greatest difference between pSM2.shCasp8.2 and pSM2.shControl transfected wells. Data are means of two replicates.

Thus, an assay for the assessment of shRNA^{mir}s and siRNAs on the cytotoxicity of TRAIL ligand on HeLa cells has been established and optimised to give the greatest difference between cells with a knock-down of Caspase-8 and cells with a control knock-down.

3.3.4 Confirmation of Caspase-8 mRNA knock-down

In order to confirm that the effect of transfection with shRNA^{mir}s and siRNAs on TRAIL-induced cytotoxicity is due to a knock-down of the Caspase 8 transcript, the levels of the transcript were measured using quantitative reverse-transcription PCR (qRT-PCR).

Transfection with pSM2.Casp8.1, pSM2.Casp8.2 and siCasp8 led to clear reductions in the level of Caspase 8 transcript as compared to levels of transcript in cells transfected with pSM2.shControl (Figure 3.10). The level of knock-down seen correlated with the different size of effects on TRAIL-induced cytotoxicity when cells are transfected with the same constructs. Transfection with siCasp8, which reduced the Caspase 8 transcript to 10% of control had the largest effect on TRAIL-induced cytotoxicity, with siCasp8 transfection increasing the survival of TRAIL treated cells from 23% to 84%. Transfection with pSM2.Casp8.2, which reduced the transcript to 20% of control levels, had a smaller effect on TRAIL-induced cytotoxicity, where transfection increased the survival of cells after treatment with TRAIL from 38% to 76%; whereas, transfection of pSM2.Casp8.1, which only reduced transcript levels to 34% of control, had little or no effect on the survival of cells treated with TRAIL (Figure 3.8a and Figure 3.8b). These results demonstrate that transfection with pSM2.Casp8.1, pSM2.Casp8.2 and siCasp8 does lead to a reduction in Caspase 8 levels and therefore that the effect on TRAIL-induced cytotoxicity is very likely caused by this reduction.

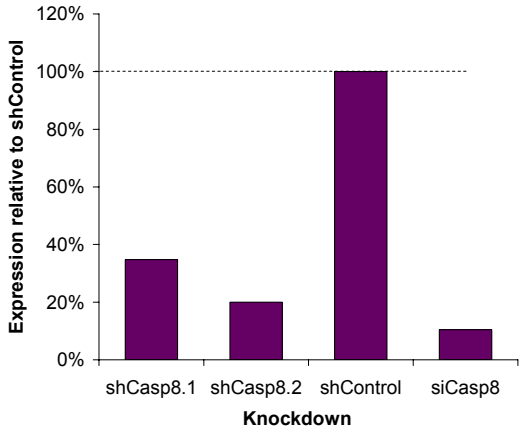

Figure 3.10 qRT-PCR confirmation of Caspase 8 knock-down
RNA was prepared from cells transfected with pSM2.shCasp8.1, pSM2.shCasp8.2, pSM2.shControl or siCasp8. RNA was reverse transcribed to generate cDNA. SYBR green qPCR was carried out on each sample using primers designed to amplify a section of the Caspase 8 transcript or primers designed to amplify a section of the ARSA transcript as control. Amplicons were designed to include large introns in the genomic sequence so that primers would amplify from cDNA but not genomic DNA. Relative levels were calculated using the Pfaffl method (Pfaffl 2001) with ARSA levels used to normalise between samples. Dashed line indicates 100% expression compared to cells transfected with pSM2.shControl.

3.4 Comparison of siRNA and shRNA^{mir} performance

3.4.1 Comparison of the effect of siRNAs and shRNA^{mir}s against Caspase-8 on TRAIL induced cytotoxicity

To compare the effectiveness of shRNA^{mir}s and siRNAs, cells were transfected with either pSM2.shCasp8.2 or siCasp8 and an appropriate non-targeting control, and the effect of treatment with a range of concentrations of TRAIL was tested. Cells were transfected with pSM2.shRNA^{mir} constructs using both a reverse transfection protocol and a traditional transfection protocol. Experiments were carried out in triplicate on one plate, and each experiment was repeated with four separate aliquots of cells, defrosted and grown independently. When cells were transfected with pSM2.Casp8.2 using a reverse transfection protocol, there was a small reduction in TRAIL-induced cytotoxicity (Figure 3.11**a**) at each concentration tested, with survival of cells after treatment with 1μg/ml TRAIL being increased from 29% for control transfected cells, to 40% for pSM2.Casp8.2 cells — a 38% increase. When cells were transfected using a traditional transfection protocol, there was a larger reduction in cytotoxicity, with a larger proportion of cells transfected with pSM2.Casp8.2 surviving treatment with all concentrations of TRAIL (Figure 3.11**b**). At 1μg/ml TRAIL, 51% of pSM2.Casp8.2-transfected cells survived, compared to 28% of control-transfected cells — a 92% increase. However, the siCasp8 siRNA performed better than the shRNA^{mir}s under all conditions (Figure 3.11**d**), with 96% of siCasp8-transfected cells surviving treatment with 1μg/ml TRAIL compared with 29% of control-transfected cells — a 274% increase. One explanation for why the shRNA^{mir}s may perform less well than the siRNA is a difference in transfection efficiency between the shRNA^{mir}s and the siRNAs. Selection of pSM2-transfected cells using the puromycin marker on the vector does not improve the proportion of cells transfected; however, an increase in effective transfection efficiency can be achieved by co-transfecting with the pIRES-P vector, which contains a functional puromycin resistance gene (Figure 3.11**c**). To increase the transfection efficiency of the pSM2 transfected cells, cells were co-transfected with pIRES-P and either pSM2.shCasp8.2 or pSM2.shControl, and puromycin resistant cells were selected before assessment of TRAIL-induced cytotoxicity. This did not increase the survival of cells transfected with pSM2.shCasp8.2 when treated with TRAIL, with 39% of pSM2.Casp8.2 transfected cell surviving. However, the relative increase in survival after TRAIL treatment between cells transfected with pSM2.shCasp8.2 and those transfected with pSM2.shControl was greatly increased and was similar to the fold difference in survival between cells

transfected with siCasp8 and those transfected with siGL2, with 328% more cells surviving after pSM2.Casp8.2 transfection than in control transfection. This is due to a decrease in the survival of cells transfected with pSM2.shControl (Figure 3.11**c**), with only 9% of cells surviving treatment with 1µg/ml TRAIL.

It can therefore be concluded that siRNAs are more effective than shRNA^{mir}s at altering the function of the TRAIL-induced cytotoxicity pathway. This difference can be reduced in terms of dynamic range, but not in terms of absolute magnitude, by co-transfecting with a puromycin resistance marker and selecting puromycin resistant cells.

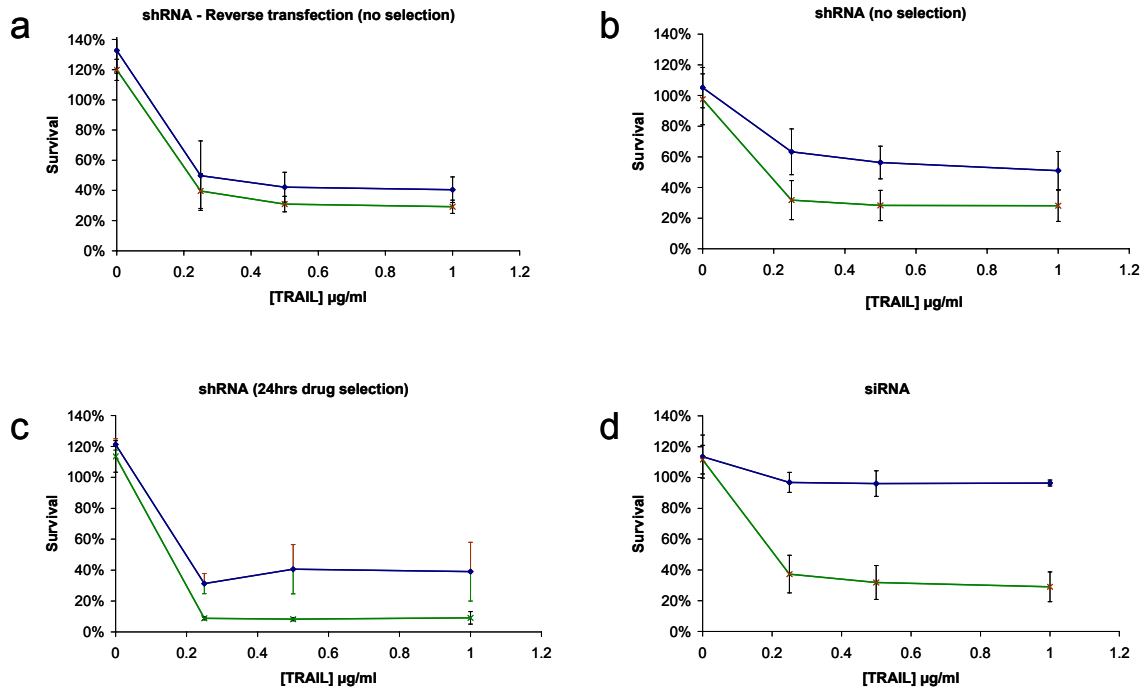

Figure 3.11 Comparison of the effects of shRNA- and siRNA-mediated knock-down of Caspase 8 on TRAIL-induced cytotoxicity.
Cells were transfected with shRNAs or siRNA targeting Caspase 8 (blue line) or a negative control (Green line) and sensitivity to TRAIL was assessed by measuring viability using alamarBlue and treating cells with a range of TRAIL concentrations. Viability was reassessed 24 hours later. a) 4,000 cells were seeded and simultaneously transfected with 80ng of shRNA^{mir} expressing construct using a reverse transfection protocol. TRAIL sensitivity was assessed 48 hours later. b) 3,000 cells were seeded and grown for 24 hours. Cells were transfected with 80ng of shRNA^{mir} constructs. TRAIL sensitivity was assessed 48 hours later. c) 12,000 cells were seeded and grown for 24 hours and transfected with 40ng of shRNA^{mir} and 40ng of pIRES-P. After 24 hours, transfected cells were selected by treatment with 2µg/ml puromycin. TRAIL sensitivity was measured 48 hours later. d) 3,000 cells were seeded and grown for 24 hours. Cells were transfected with 2.5pmol siRNA. TRAIL sensitivity was assessed 48 hours later. All points are means of four biological replicates. Error bars represent 1 standard deviation

3.4.2 Blind pseudo-screens show a clone-by-clone screen is practical with siRNA, but not shRNA^{mir}s

Although siRNAs targeting Caspase 8 have a larger effect on cytotoxicity induced by treatment with the TRAIL ligand than shRNA^{mir}s targeting the same gene, this does not imply that the siRNA mediated effect is large enough to allow an effective screen, or that the

shRNA^{mir} mediated effect is not large enough. The feasibility of performing clone-by-clone screens using both shRNA^{mir}s and siRNAs was examined by using a blind pseudo-screen. Here, siRNAs/shRNA^{mir}s were arrayed in a 96-well plate by a colleague in such a way that the plate contained 5-15 wells with siRNAs/shRNA^{mir}s targeting a positive control (Caspase 8 in this case) and all other wells filled with negative controls. Without knowledge of the location or number of the positive controls on the plate, the constructs were transfected into a 96-well plate of cells and the level of cytotoxicity induced by treatment with the TRAIL ligand was assayed. If the effect of the knock-down is large enough to allow screening, it should be possible identify the number and position of wells containing positive controls. As well as measuring the success of this experiment in terms of sensitivity and accuracy, a Z' score (see section 3.1.3) can be calculated and used to compare different protocols.

Blind pseudo-screens were carried out using shRNA^{mir}s, shRNA^{mir}s co-transfected with pIRES-p and selection applied for 48 hours with puromycin, or using siRNAs. When shRNA^{mir}s were used 70% of the positive controls were identified, while 60% of the wells selected were false positives. This was improved by using puromycin to select cells co-transfected with pIRES-p, allowing 77% of the positives to be selected without the selection of any false positives. In contrast, 100% of wells transfected with the siRNA positive control were selected without the selection of any false positives. The difference is even greater when Z' factors, calculated from the values of all wells on the plate, are considered. The Z' factors were -.06, -0.7 and 0.46, for shRNA^{mir}s without selection, shRNA^{mir}s with selection and siRNAs, respectively (Figure 3.12). Z' scores of less than 0 are generally thought to indicate an assay of limited use, while those with a Z' prime score of greater than 0.5 are thought to be excellent assays by those involved in chemical screening (Zhang, Chung & Oldenburg 1999).

Thus siRNAs targeting Caspase 8 outperform shRNA^{mir} targeting the same gene by a wide margin when considering Z' scores, with the Z' score for siRNAs coming close to that thought to indicate an excellent assay (Zhang, Chung & Oldenburg 1999). The consequence is seen in the number of positives picked out in the blind pseudo-screen. From this it can be concluded that screening with siRNAs is feasible and offers the possibility of an accurate and sensitive screen for genes involved in TRAIL-induced cytotoxicity. In contrast, shRNA^{mir}s, whether or not drug selected, do not provide the necessary power to distinguish genes that affect TRAIL-induced cytotoxicity compared to those that have no effect, particularly since Caspase 8 was the gene that had the largest effect on TRAIL-induced cytotoxicity in previous screens (Aza-Blanc et al. 2003).

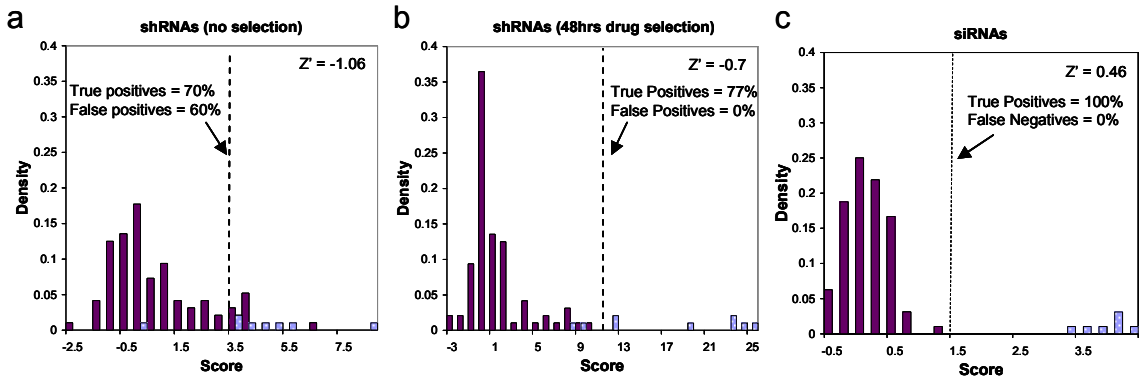

Figure 3.12 Histograms showing distributions of positive and negative controls in blind pseudo-screens. Cells were seeded and grown for 24 hours then transfected with shRNAmir expressing vectors (a), co-transfected with pIRES-P and shRNAmir expressing vectors (b) or siRNAs (c), targeted against either Caspase 8 or a negative control. The identity of the siRNAs/shRNAmirs was unknown to the experimenter (see text). Cells were either grown for 48 hours and assessed for sensitivity to 1μg/ml TRAIL (a and c) or grown for 24 hours and then treated with 2μg/ml puromycin for 48 hours before being assessed for sensitivity to 1μg/ml TRAIL. Standardised scores were calculated for each well using robust estimates for μ and σ. An experimenter selected cut off for hits was selected by manual inspection. (dashed line). After positions of positives were revealed, Z' factors between positive controls (light bars) and negative controls (dark bars) were calculated using robust estimators for μ and σ.

3.4.3 Differences in assay outcome between siRNAs and shRNA^{mir}s are due to different knock-down efficiencies for a wide range of positive controls

Until this point all experiments have examined only the effect of knocking down one gene – Caspase 8. To study the effects of knocking a larger selection of genes, 18 genes that have previously been implicated in TRAIL-induced cytotoxicity (see Table 1-2) and were present in the Expression Arrest (v1.3) library, were selected. Between two and four siRNAs targeting each of these genes were obtained. The effect of transfecting each of these siRNAs into HeLa cells on TRAIL-induced cytotoxicity was assessed. In total, 11 of the genes had at least one siRNA that had a significant effect on TRAIL-induced cytotoxicity; of these, four had two siRNAs that had a significant effect (Figure 3.13). For each gene, the siRNA that had the greatest effect was selected. These siRNAs were transfected into cells and tested for their effect on TRAIL sensitivity together on one plate. This was compared to the effect of co-transfecting pIRES-P with pSM2 constructs from the Expression Arrest library targeting the same genes, and selecting for puromycin resistant cells, on TRAIL-induced cytotoxicity. In total, nine (TNFSR10A, Caspase 3, PRKRIR, FBXO11, PRKCQ, SMAC, ABL2, BID and Caspase 8) out of the 18 genes tested had an siRNA that significantly increased the survival after TRAIL treatment. In contrast, the only pSM2 construct that had a significant effect on TRAIL-induced cytotoxicity was pSM2.shCasp8.2 (Figure 3.14)

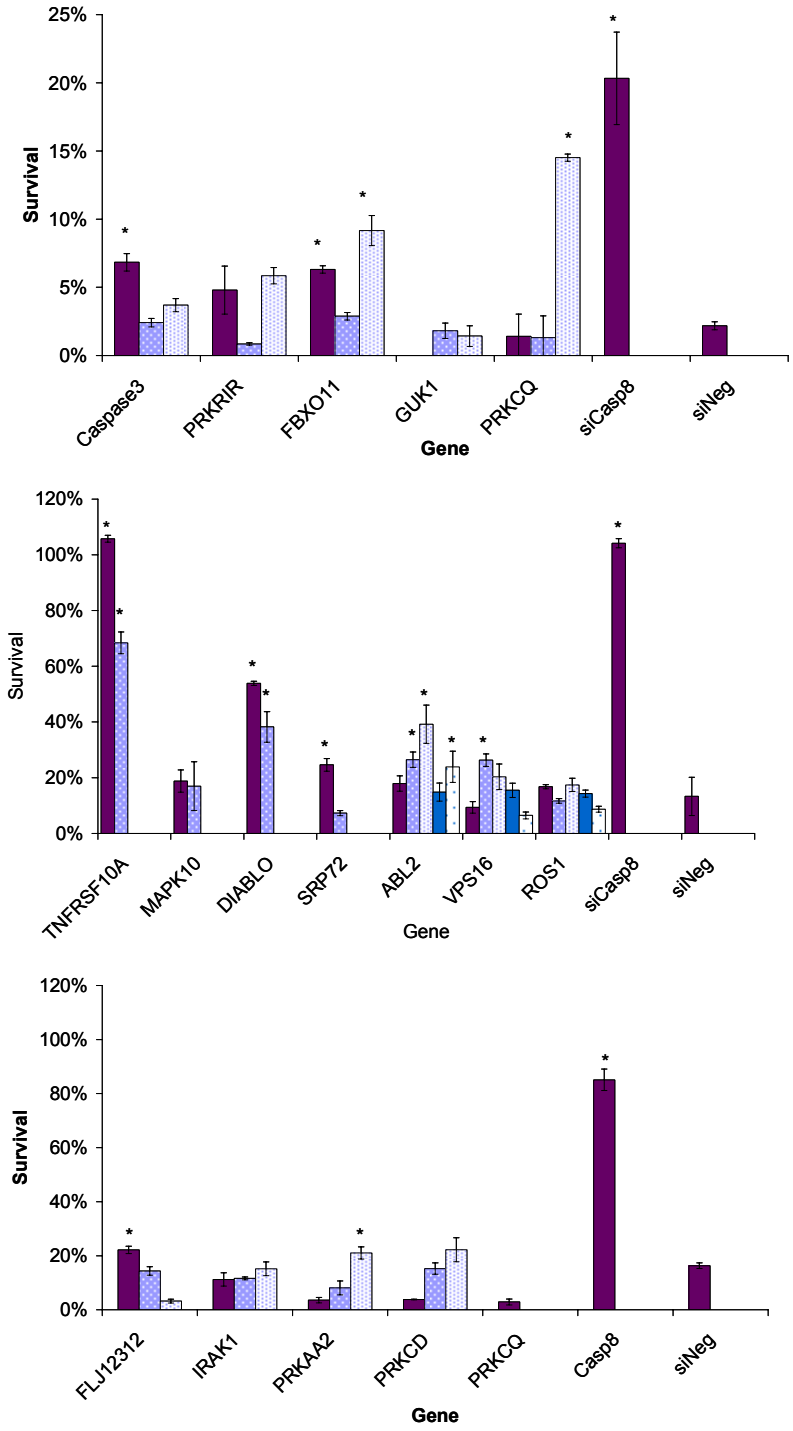

Figure 3.13 Effects of knock-down of 18 positive controls on TRAIL-induced cytotoxicity.
Cells were transfected with between two and four siRNAs targeting 18 genes previously implicated in the TRAIL-induced cytotoxicity pathway, or a non-targeting siRNA (siNeg) on three separate plates. Cells were assessed for sensitivity to 1μg/ml TRAIL 48 hours later. Each shaded bar represents a different siRNA targeting the gene indicated. First results for each of ABL2, VPS16 and ROS1 represent pools of the other four siRNAs for that gene. Results are the mean of three repeats. Error bars represent 1 standard deviation. * results significantly different from negative control using Student's t-test on log transformed data (Bonferroni corrected $\alpha = 0.05$).

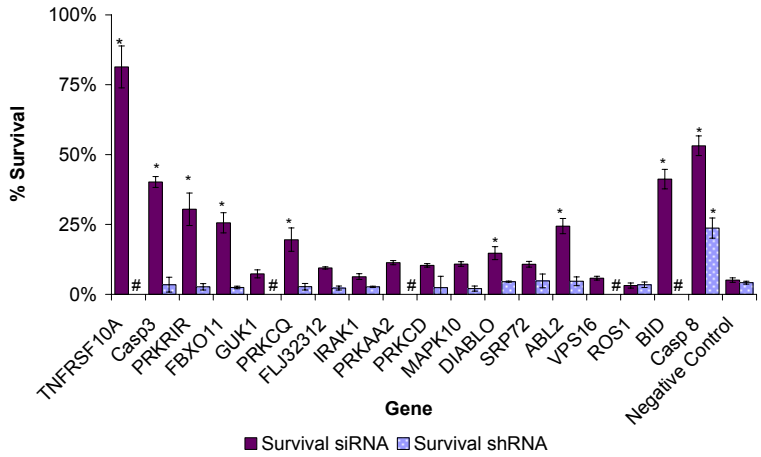

Figure 3.14 Comparison of effect of knock-down of 18 positive controls by siRNA or shRNA^{mir} on TRAIL-induced cytotoxicity.
Cell were either transfected with the best siRNA targeting one of 18 genes previously implicated in TRAIL-induced cytotoxicity or co-transfected with pIRES-P and a vector expressing an shRNA^{mir} targeting the same gene from the Expression Arrest library. siRNA-transfected cells were assessed for TRAIL sensitivity 48 hours later. pIRES-P/shRNA-transfected cells were grown for 24 hours and then selected with 2μg/ml for 48 hours before sensitivity to 1μg/ml TRAIL was assessed. Data are means of three replicates. Error bars represent 1 standard deviation. * results significantly different from negative control using Student's t-test on log transformed data (Bonferroni corrected α= 0.05). # vector did not contain expected hairpin upon sequencing.

In order to find the source of this difference, qRT-PCR was used to measure the change in expression levels of the targeted transcript when siRNA/shRNA^{mir}s were introduced into the cells. Oligonucleotide primers were designed to amplify from the cDNA of each transcript and not from genomic DNA by either including at least one large intron in the amplicon, or spanning an intron/exon boundary with one oligonucleotide primer. Primers were tested to ensure they amplified one and only one fragment and their efficiency measured as described by Pfaffl *et al.* (Pfaffl 2001). Primers were rejected if efficiency was less than 80% or greater than 110% (Pfaffl 2001). Primer pairs that failed were redesigned up to three times. Oligonucleotide primers were successfully designed for 13 genes (see Appendix B). Transfection of siRNAs targeting eight of the 13 genes (61%) caused a reduction in expression levels of the targeted transcript to 30% or less of the level measured when an siRNA was transfected which targets no transcript. Transfection of vectors encoding hairpins targeting three out of 10 genes (30%) led to a similar reduction in transcript levels. There were eight cases where transfection of an siRNA led to a significant reduction in TRAIL-induced cytotoxicity but transfection of a hairpin encoding vector targeting the same gene did not. In all but one of these cases transfection of the siRNA led to a greater reduction in transcript levels than transfection of the hairpin-encoding vector (Figure 3.15).

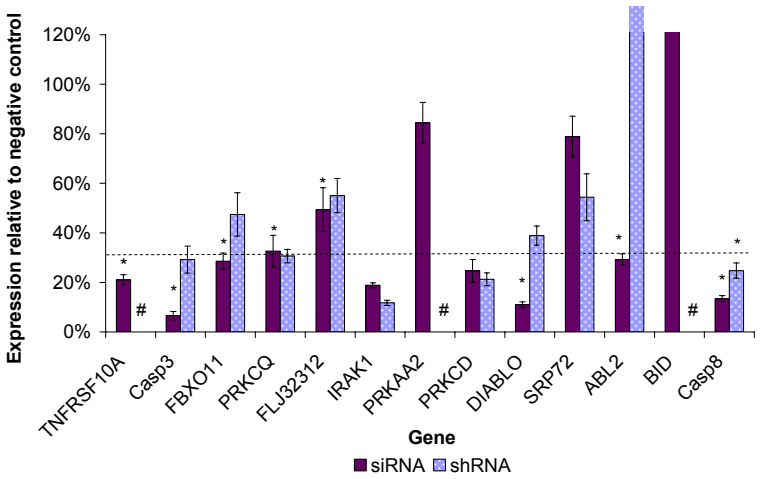

Figure 3.15 qRT-PCR measurement of knock-down by siRNAs/shRNAmirs targeting 13 positive controls

RNA was prepared from cells either transfected with the best siRNAs targeting one of 13 genes previously implicated in TRAIL-induced cytotoxicity or co-transfected with pIRES-P and vectors expressing an shRNA^{mir} targeting the same genes and selected for 48 hours with 2μg/ml puromycin. cDNA was produced by reverse transcribing RNA. SYBR green qPCR was carried out using primers designed to amplify a section of the 13 genes targeted, the GAPDH gene and the ACTB gene. Primers were designed to amplify from cDNA only. Expression levels relative to negative controls were calculated using a variation of the Pfaffl method to allow normalization to multiple housekeeping genes using GAPDH and ACTB to normalise samples (Hellemans et al. 2007). * siRNA/shRNA elicited a significant change in TRAIL sensitivity assays. # vector did not contain expected hairpin upon sequencing. . The dashed line represents 30% expression relative to control.

In an attempt to explain the relatively poor performance of the hairpin-encoding vectors, the hairpins used were sequenced. Although the constructs in the expression arrest library are supposed to be sequence-verified, five of the constructs sequenced did not contain the expected hairpin sequence (vectors which were predicted to express hairpins targeted against TNFRSF10A, GUK1, PRKAA2, VPS16 and BID). The results from these genes are not shown in the above results, or included in any calculations or conclusions. Thus in fact shRNA^{mirs} targeting only 13 genes were tested for effects on the TRAIL induced apoptosis, and the effect of only 10 shRNA^{mir}s on levels of the targeted transcripts was measured.

3.5 Conclusion

An assay for the effect of the TRAIL ligand on the viability of HeLa cells has been established. It has been demonstrated that treatment of HeLa cells with the TRAIL ligand leads to cytotoxicity. It has further been demonstrated, by the knock-down of the key TRAIL pathway gene Caspase 8, that TRAIL-induced cytotoxicity can be modulated using both shRNA^{mir}- and siRNA-mediated RNAi against genes involved in the pathway. The optimisation experiments undertaken emphasise the importance of carefully examining the

effect of as many different variables on the outcome of the assay, not only separately but together. For example, decreasing the cell density at the beginning of the assay increases the sensitivity of the cells to TRAIL-induced cytotoxicity (Figure 3.6), but decreases the effect of using RNAi to knock-down genes involved in the TRAIL pathway (Figure 3.9 and Figure 3.8b).

The final assay conditions selected from the optimisation process were to seed 3,000 cells and grow for 24 hours. For siRNA experiments, the cells are transfected with 2.5pmol of siRNA using 0.12µl of Lipofectamine 2000. For shRNA^{mir} experiments, cells are transfected with 80ng of plasmid DNA using 0.24µl of siPort XP-1. Cells are then grown for 48 hours. The viability of cells is assessed using alamarBlue before treatment with 1µg/ml TRAIL in serum-free media for 24 hours. The viability of cells is then assessed again using alamarBlue and the percent survival of the cells is calculated using Equation 6. Using these conditions, 28% of cells transfected with pSM2.shControl and 29% of cells transfected with siGL2 survive TRAIL treatment. In contrast, 51% of cells transfected with pSM2.shCasp8.2 and 96% of cells transfected with siCasp8 survive (Figure 3.11). This compared with an average survival after treatment with TRAIL of 38.5% of negative control-transfected cells reported in previous, similar work (Aza-Blanc et al. 2003), showing that the assay presented here was at least as sensitive as that of Aza-Blanc *et al.*.

The effect of using siRNA- and shRNA^{mir}-mediated RNAi against Caspase-8 on the levels of TRAIL-induced cytotoxicity was compared, both by directly comparing the survival rates of cells treated with a range of TRAIL concentrations and using blind pseudo-screens to assess real performance in a screening situation.

In the direct comparison, siRNA-mediated knock-down of Caspase-8 outperformed shRNA^{mir}-mediated knock-down of Capase-8, with a greater difference between control and Capase-8 knock-downs being seen using siRNA-mediated knock-down at all concentrations of TRAIL tested. This was true when either the absolute difference or the fold change in survival after TRAIL treatment was considered. One possible reason for this difference could be a difference in transfection efficiency. Co-transfecting with shRNA^{mir}-encoding vectors and a plasmid carrying a puromycin resistance marker and selecting transfected cells using puromycin increased the effective transfection efficiency, as measured by the number of transfected cells compared to the total number of living cells. However, using selection did not improve the absolute difference in TRAIL-induced cytotoxicity between cells transfected with a negative control, and those transfected with a construct targeting Caspase-8, although the fold change in survival between negative control and the Caspase-8 knock-

down was increased. Selection using the marker encoded on the pSM2 vector itself proved not to be effective. The reasons for this remain unknown, although one difference between the puromycin resistance marker on pIRES-P and pSM2 is the promoter used to transcribe the gene, with the marker on pIRES-P being transcribed from a CMV promoter and the marker on the pSM2 vector being transcribed from a PGK promoter.

The differences seen in the direct comparison were reflected in the performance observed in the blind pseudo-screens. Screens performed using shRNA^{mir}s targeting Caspase-8, either selected or unselected, gave less than perfect results with sensitivities of 70% and 77% and false positive rates of 60% and 0% for the selected and unselected protocol, respectively. This reflects the finding from the direct comparisons, where selecting for transfected cells reduced the background noise, but did not increase the absolute size of the positive signal. The Z'-factors for these screens were -1.06 and -0.7, respectively. These are both below the minimum Z'-factor of 0 thought necessary to perform a successful screen (Zhang, Chung & Oldenburg 1999). In contrast, pseudo-screens performed using siRNAs found 100% of the positive controls with no false positives. Here the Z'-factor was 0.42, well above 0 and close to the 0.5 recommended for an "excellent" assay.

The analysis was extended for a further 17 genes previously linked to TRAIL-induced apoptosis. siRNA-mediated knock-down against 50% of these led to a significant reduction in TRAIL-induced cytotoxicity, including all five genes tested involved in the core death pathway (Caspases 8 and 3, the death receptor TNFS1R10A, BID and SMAC). In contrast, shRNA^{mir}-mediated knock-down lead to a significant change in TRAIL-induced apoptosis in only one case: Caspase 8. The failure of several of these vectors can be attributed to the fact that sequencing revealed that they did not contain the expected hairpin sequence, which raises an issue as to the integrity and accurate annotation of the library. However, even when this problem is taken into account, the conclusion that chemically synthesised siRNAs performed better than the shRNA expressing vectors contained in the expression arrest library still holds. One explanation of these results is provided by measuring the levels of targeted transcripts using qRT-PCR. In all but one case, where an siRNA produced a significant reduction in TRAIL-induced cytotoxicity, but the shRNA^{mir} targeting the same gene did not, the siRNA reduced the levels of the transcript further than the shRNA^{mir}.

. Taken together, these results show, that in the context of a screen for genes that alter the sensitivity of cells to TRAIL-induced apoptosis, the commercially designed and purchased siRNAs are more powerful than the shRNA^{mir}s expressed from the clones in the Expression Arrest library. Not only do the blind pseudo-screen results demonstrate that

shRNA^{mir}-mediated knock-down of Caspase 8 does not provide a large and reliable enough difference from control transfected cells to allow a large-scale screen, but knock-down of no other gene using constructs from the Expression arrest library gives a significant change in TRAIL-induced cytotoxicity. The reasons for this, beyond the fact that the shRNA^{mir}-encoding vectors do not induce such a large reduction in transcript levels, remain unknown. There are three possible reasons why this might be the case. Firstly, the shRNA^{mir} may not being expressed at a high enough level in a large enough number of cells. Secondly, they may not be being properly processed to give active siRNAs. Thirdly, the shRNA^{mir}s are being expressed and processed, but are not inducing degradation of the targeted mRNA. In the first case, the fact that selecting for transfectants does not eliminate the difference suggests that the raw transfection efficiency is not the reason behind the differences. However, this does not rule out the amount of plasmid being delivered or the level of transcription from the transfected plasmid being the cause of the difference in performance. In the third case, it should be noted that the sequence of the shRNA^{mir} and the siRNAs were not generated using the same algorithm. In this case, it would not simply be a case of shRNA^{mir}s being less powerful than siRNAs, but one of these shRNA^{mir}s being less powerful than these siRNAs.

.Screens may be performed either in a clone-by-clone manner, with each well on a 96- or 384-well plate transfected with one or multiple siRNA(s)/shRNA^{mir}-encoding vector(s) targeting one gene, or in a pooled manner, where hairpin-encoding vectors targeting multiple genes are transfected into a population of cells. A selection is applied to the population of cells and the shRNA^{mir}s present in the surviving cells are determined, based on the assumption that shRNA^{mir}s that allow cells to escape the selection will be over-represented in the post selection population. Such a strategy could reduce the cost and increase the throughput of screening. In an ideal assay, the selection applied would be strong enough to allow only cells expressing hairpins targeting genes involved in the process in question to escape. However, this is not the case for in the assay as optimised here with a relatively large number of negative control transfected cells surviving treatment with TRAIL. Together with the results on the poor performance of shRNA^{mir}s, this suggests that adopting a pooled screen strategy would be neither efficient nor successful in this case. It should also be noted that preparing transfection quality DNA from the full library would be an expensive and time consuming process (eliminating one of the supposed benefits of shRNA^{mir} based approaches – cost). Therefore, at least in the context of the assay for TRAIL-induced cytotoxicity presented here, screens should be performed using libraries of arrayed siRNAs rather than shRNA^{mir} expressing vectors.