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TARGETS OF ELF5 IN MOUSE TROPHOBLAST STEM CELLS

A thesis

submitted in partial fulfilment

of the requirements for the degree

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ABSTRACT

The Placenta is an essential organ for all mammalian embryonic development as it provides the nutritional link between maternal and foetal blood streams. The cells which go on to proliferate and contribute to all the major cell types of the embryo derived placenta have been located to the trophoblast (TE) cells overlying the Inner Cell Mass (ICM) of the embryo. Immortal cell lines have been subsequentially derived from this tissue and called Trophoblast Stem (TS) cells. In parallel with their *in vivo* counterparts they are also reliant on Fibroblast growth factor 4 (FgF4) (Tanaka *et al.*, 1998) and Activin/Nodal signalling (Guzman-Ayala *et al.*, 2004).

The Ets family transcription factor, Elf5, has been shown to be specifically expressed in the early placental trophoblast and subsequent derived tissues. Mice deficient in Elf5 failed to form a placenta post implantation. Furthermore TS cells were unable to be derived from *Elf5* knockout embryos (Donnison *et al.*, 2005). This work suggested that *Elf5* plays an essential role in TS cells and their differentiation. The aim of this study was to determine the downstream target genes of Elf5 in mouse TS cells. The target genes of Fgf4 and Activin/Nodal signalling in TS cells were also investigated. This work is hoped to contribute to an overall greater understanding of the molecular networks underlying TS cell maintenance and to contribute to our knowledge of early placental development.

Small interfering RNA (siRNA) targeted reduction of *Elf5* mRNA expression in mTS cells was achieved using two independent siRNAs; with *Elf5* reduction exceeding 80%. The resulting changes in gene expression were measured in order to determine the downstream targets of *Elf5*. Selected genes known to be important for trophoblast differentiation and maintenance were measured using real-time PCR in a candidate gene approach .Global changes in gene expression as a consequence of Elf5 silencing were measured using an Affymetrix microarray. Global changes in gene expression due to growth factor (Fgf4 and/or Activin) removal were also measured. Expression of 22 genes was changed using either of the *Elf5* siRNA oligonucleotides. Of these, 9 were also significantly changed by growth factor removal. Included in this set were *Synopl*,

Hst3st3b1, Cyr61 and *Sox2*. In the overall analysis, many genes whose expression changed upon loss of Elf5 are known to play important roles in trophectoderm cell specification. Real-time PCR validation agreed closely with the up or down regulation measured using the microarray. This work has thus led to the discovery of sets of Elf5 target genes potentially involved in trophoblast stem cell function and has provided the foundation for future work exploring the molecular pathways of trophoblast development.

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ABBREVIATIONS

AVE	Anterior visceral endoderm	PBS	Phosphate buffered saline
CMV	Cytomegalovirus	PCR	Polymerase chain reaction
cDNA	Complementary DNA	TRE	Tetracycline response element
Ct	Crossing threshold	qRT-PCR	Quantitative reverse transcription polymerase chain reaction
DMSO	Dimethyl Sulphoxide	RLU	Relative light intensity
EPI	epiblast	RMA	Residual means analysis
ES	Embryonic stem cells	RNAi	RNA interference
ExE	Extra-embryonic ectoderm	RT	Reverse transcription
F4H	Fibroblast growth factor 4 and heparin	siRNA	Small interfering RNA
FACS	Fluorescent activated cell sorting	TA	Tetracycline activator
FC	Flow cytometry	TE	trophectoderm
FCS	Foetal calf serum	rtTA	Reverse tetracycline activator
Fgf4	Fibroblast growth factor 4	TRE	Tetracycline response element
ICM	Inner cell mass	TS + A	Trophoblast stem cell medium supplemented with Actvin
IVT	*In vitro* transcription	TS + F4H	Trophoblast Stem

			cell medium supplemented with Fgf4 and Heparin
MEF	Mouse embryonic fibroblast	TS only	Trophoblast stem cell medium without any growth factors.
MM	Master mix		
mRNA	Messenger RNA		
mTS	Mouse trophoblast stem cells		

CHAPTER 1 : LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

The placenta is the essential organ for all mammalian development in the uterine environment as it mediates the interaction between the growing foetus and the maternal blood stream. Indeed, any genetic or environmental insult that affects the development of the placenta can lead to placental insufficiency, fetal growth retardation and death. Formation of the placental lineage has occurred by the blastocyst stage in the mouse embryo when two clearly defined lineages are seen; the Inner cell mass (ICM) which goes on to become all cell lineages of the embryo proper and the trophectoderm (TE) which proliferates and differentiates into extra-embryonic ectoderm tissue and is essential for the formation of all embryo derived placental cell types (Rossant & Cross, 2001). The proliferative cells of the trophectoderm are in close contact with the ICM and ICM derived tissues, and have been isolated as an immortal line of cells called trophoblast stem cells (TS cells) (Tanaka *et al.*, 1998). These cells have been found to be reliant both *in vitro* and *in vivo* on growth factors such as Fibroblast growth factor 4 (Tanaka *et al.*, 1998) and Nodal (Guzman-Ayala *et al.*, 2004). The Ets transcription factor *Elf5* has been shown to play an essential role in early placental development (Donnison *et al.*, 2005). *Elf5* homozygous mutant mice failed to develop the extra-embryonic ectoderm, and TS cells could not be derived from mutant blastocyts even though they were correctly specified. These results suggest *Elf5* is essential for the maintenance of TS cell and extra-embryonic ectoderm precursors . Although several transcription factors are known to be specifically expressed in the trophectoderm, little has been done to investigate the molecular nature of their interactions in TS cells on a global genome scale or the probable over lapping molecular networks with Fgf4 and Nodal/Activin signalling; also so important to the trophectoderm .

1.2 MOUSE TROPHOBLAST DEVELOPMENT

1.2.1 Mouse Pre-Implantation Development

At the early blastocyst stage of the mammalian embryo two morphologically distinct cell types are seen (Figure 1.2-1): the trophectoderm (TE), which is an epithelial outer

layer of cells that contributes solely to the embryo-derived placental tissues; and the inner cell mass (ICM), cells which become the embryo and contribute to extraembryonic endoderm and mesoderm (Kunath *et al.*, 2004). The formation of the blastocyst, and hence of a new individual begins at fertilisation. Following fertilisation the zygote undergoes three rounds of cleavage division to form the eight cell stage. At this stage the embryo undergoes compaction; the blastomeres become polarised and express the membrane binding protein E-Cadherin (Kunath *et al.*, 2004). Cellular division can then give rise to two types of daughter cells; asymmetric division gives an outer polar cell and an inner non-polar cell, while symmetric (radial) division results in two polar daughter cells (Johnson & Ziomek, 1981). If the embryo is left undisturbed the outer cells go on to form the trophectoderm; so it is though that the inheritance of different apical and basal regions leads to differences in cell fate (the cell polarity model) (Yamanaka *et al.*, 2006). However, at the eight cell stage all cells retain the ability to form either trophectoderm or ICM because experiments involving positional changing of the blastomeres at this stage show they are still able to contribute to both lineages (Rossant & Vijh, 1980; Ziomek *et al.*, 1982). By the 16-cell stage first lineage decision has been made and cells no longer have the same plasticity (Ziomek *et al.*, 1982).

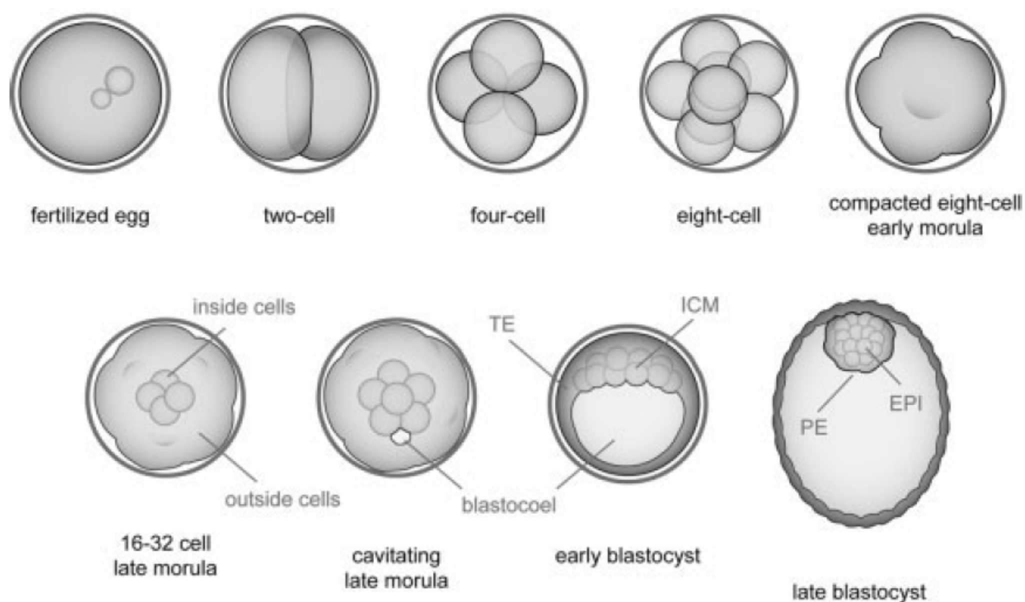


Figure 1.2-1: Mouse Pre-implantation development.
(source: (Yamanaka et al., 2006)

The ball like structure of the blastocyst is formed by embryonic day (E) 3.5 in the mouse, as the outer TE cells pump fluid internally and form the blastocoel cavity, with the ICM lying to one side (Rossant & Cross, 2001). In the mouse a stem cell population (trophoblast stem cells) has been isolated from the outer cells of the blastocyst in the presence of Fgf4 and an embryonic fibroblast cell feeder layer (Tanaka *et al*., 1998). These cells have been shown to contribute solely to the placental cell lineages *in vivo*. The cells of the ICM have also been captured in immortal embryonic stem (ES) cell lines (Solter, 2006). A day later the mouse embryo is beginning to implant, and the ICM and TE have become several distinct cell types. The ICM has formed the epiblast (EPI) and the primitive endoderm and the TE is made up of the polar trophectoderm which overlies the ICM, and the mural trophectoderm (Rossant & Cross, 2001).

1.2.2 Mouse Placental Development

The placenta is the first organ to develop and performs a myriad of roles besides exchange of nutrients and wastes between the foetus and mother, including altering metabolic, endocrine, cardiovascular and immune functions of the mother (Cross, 2005). The early derivatives of the trophectoderm also play an essential role in patterning and correct development of the embryo (Donnison et al., 2005; Georgiades & Rossant, 2006; Richardson *et al*., 2006). The embryonic portion of the placenta is formed from derivatives of both the ICM and TE. The specialised cell types derived from the polar and mural trophectoderm of the blastocyst play different roles in placental invasion and function and are formed in a hierarchical way. Sequential interactions of transcription factors give unique patterns of gene expression that cause the specification of each cell type (Figure 1.2-2).

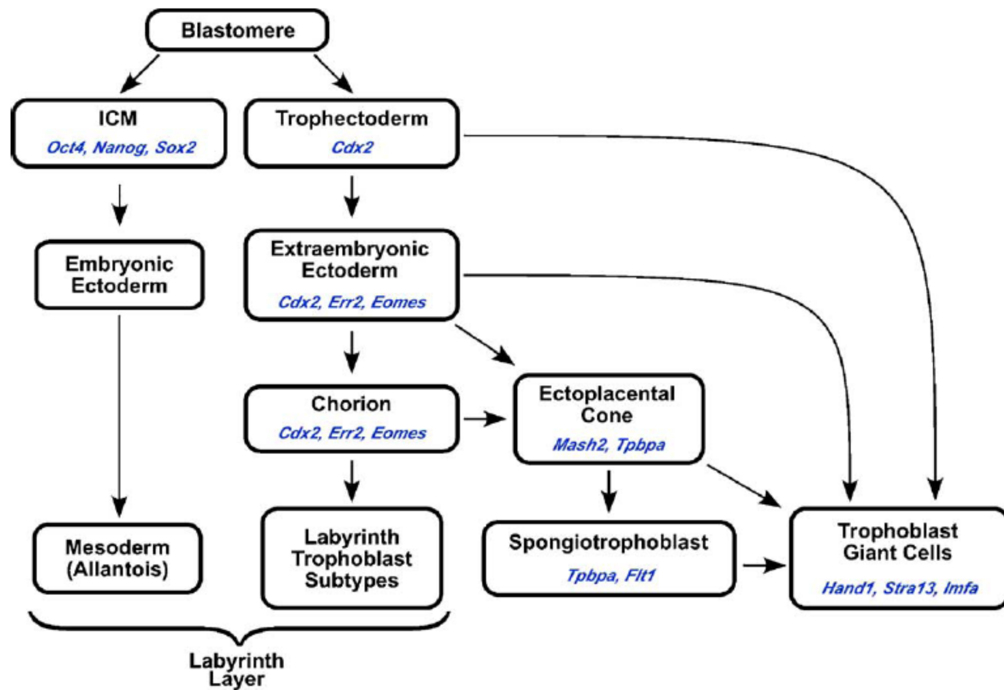


Figure 1.2-2: The different subtypes of the embryo derived placenta and expression of some of the genes important for their specification and maintenance (source: (Simmons & Cross, 2005).

The first terminal cell type of the placenta to form are giant cells derived from the mural trophectoderm. Giant cells derived from the mural trophectoderm are called primary giant cells (Simmons & Cross, 2005) and are polyploid with large nuclei due to endoreduplication of their DNA. Giant cells express tissue remodelling genes (e.g matrix metalloproteinases) and mediate invasion of the conceptus into the maternal decidua. They produce vasodilators (e.g. vascular endothelial growth factor) and angiogenic factors (e.g. proliferin) as well as hormones (e.g. placental lactogen 1 [*Pl1*]) (Carney *et al.*, 1993) to promote maternal blood flow to the implantation site and maternal responses to pregnancy (Simmons *et al.*, 2007). Giant cells require the transcription factor Hand1 for their formation (Scott *et al.*, 2000). *Hand1* knockout mice (where both copies of the *Hand1* gene have been made non-functional) die by embryonic day (E) 8.5 due to a failure in giant cell formation.

The polar trophectoderm over laying the epiblast rapidly proliferates to form the extra-embryonic ectoderm and the ectoplacental cone (Rossant & Cross, 2001) so that the embryo is elongated into a 'cup shape' with the extra embryonic ectoderm lying close to the epiblast and the ectoplacental cone further away. The proliferative maintenance

of the polar trophectoderm and subsequent extra-embryonic ectoderm is reliant on fibroblast growth factor-4 (Fgf4) and TGFβ signalling from the ICM and later the epiblast (Simmons & Cross, 2005).

Fgf4 production is restricted to the ICM of the blastocyst and later to the epiblast (Rappolee *et al.*, 1994 291), while its receptor, Fgfr2, is expressed specifically by the trophectoderm of the blastocyst, and later restricted to the extra-embryonic ectoderm (Haffner-Krausz *et al.*, 1999). *Fgf4* and *Fgfr2* null mutants have similar phenotypes; both knockouts fail just after implantation and trophoblast stem cells cannot be derived from blastocyst outgrowths, indicating Fgf4 signalling is essential for the trophectoderm lineage (Feldman *et al.*, 1995) (Arman *et al.*, 1998).

The TGFβ member, Nodal, plays an important role in anterior-posterior patterning of the embryo (Beck *et al.*, 2002) and it is also required for correct development of the placenta and maintenance of trophoblast stem cells *in vitro* (Guzman-Ayala *et al.*, 2004). Nodal is expressed by the epiblast as a pre-protein which must be cleaved before it is active. The extra-embryonic ectoderm expresses two proteases, Furin and PACE4 which cleave pro-Nodal (Beck *et al.*, 2002). Nodal then maintains TS cells by upregulating *Fgf4* expression in the epiblast and by acting directly on the extra-embryonic ectoderm to maintain expression of *Cdx2, Errβ,* and *Eomes* (extra-embryonic ectoderm markers) and to suppress *Mash2* (an ectoplacental cone marker) (Guzman-Ayala *et al.*, 2004). Activin, a protein closely related to Nodal which shares the same signalling receptors can also maintain expression of extra-embryonic ectoderm markers and prevent *Mash2* expression (Guzman-Ayala *et al.*, 2004).

The ectoplacental cone is characterised by expression of *Tpbpa* and *Mash2* (Simmons & Cross, 2005). The ectoplacental cone is thought to differentiate into secondary giant cells and spongiotrophoblast (Figure 1.2-3), since giant cells surround the outer limits of the conceptus and TS cells in culture have been shown to transiently express these markers before becoming giant cells (Carney *et al.*, 1993). The transcription factor *Mash2* is essential for development of the spongiotrophoblast (Guillemot *et al.*, 1994). However it also inhibits giant cell formation (Scott *et al.*, 2000) and is down regulated as differentiation to giant cells proceeds. Recent evidence also suggests that there is a greater diversity in secondary giant cells and that not all of them originate from the ectoplacental cone (Simmons *et al.*, 2007).

The extra-embryonic ectoderm proliferates to form the chorion and both tissues are characterised by a similar set of genetic markers. The allantois, which is derived from the extra-embryonic mesoderm, attaches to the chorion at around day E8.5 (Rossant & Cross, 2001). After this occurs, foetal blood vessels begin to develop from the allantois and invade the choiron to create the foetal derived vascular network of the placenta (Rossant & Cross, 2001). Chorionic trophoblast differentiates alongside the foetal blood vessels into densely branched villi made up of several specialised cell types including syncytiotrophoblast which are characterised by the expression of *Gcm1*(Simmons & Cross, 2005) and giant cells. The entire structure is known as the labyrinth (Figure 1.2-3) and is where nutrient exchange between foetal and maternal blood occurs. *Gcm1* mutants fail to form syncytiotrophoblast cell types and the chorion remains flat (Anson-Cartwright *et al.*, 2000). The structure of the labyrinth trophoblast is based around the maternal blood sinuses (Figure 1.2-3). Giant cells are in direct contact with the maternal blood followed by two layers of syncytiotrophoblast to form a three layered barrier adjacent to a foetal blood vessel (Cross, 2005).

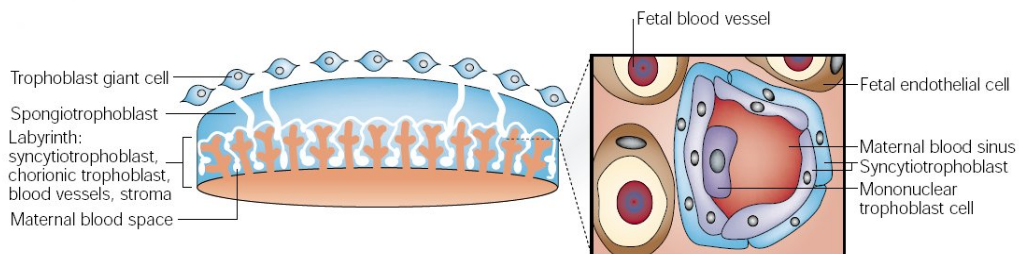


Figure 1.2-3: structure of the mouse placenta and of the trophoblast interface between maternal and fetal blood in the labyrinth. (source: (Rossant & Cross, 2001)

1.3 TS CELLS AS A MODEL FOR EARLY PLACENTAL DEVELOPMENT

1.3.1.1 Trophoblast Stem cell derivation and maintenance

Trophoblast stem cell lines are able to be derived from the outer trophectoderm cells of the blastocyst and from the resulting extra-embryonic ectoderm and chorion until the first somite pair of development, however not from the ectoplacental cone (Uy *et al.*, 2002). They recapitulate the *in vivo* situation closely in that they are also dependent

on Fgf4 and Activin/Nodal signalling (Erlebacher *et al.*, 2004; Tanaka *et al.*, 1998) to maintain their multipotent stem cell capability, and deprived of either growth factor differentiate precociously to the giant cell fate (Hemberger *et al.*, 2004; Ilgren, 1981). Because the process of differentiation is easily controlled by the removal of Fgf4 and Activin growth factors, TS cells are a powerful model system to investigate gene regulation of trophoblast differentiation (Winger *et al.*, 2007). They also exhibit a similar pattern of gene expression as the extra-embryonic ectoderm (*Cdx2, Eomes, Errβ*); and in fact the ability to derive TS cells from a knockout mouse model has become a test to see if that particular gene is required for the extra-embryonic lineage. TS cells retain the ability to differentiate *in vivo*. When injected into blastocysts, TS cells grown in embryonic fibroblast conditioned medium or medium supplemented with Activin/Nodal are able to contribute to all cell lines of the placenta but not the embryo itself (Erlebacher et al., 2004; Tanaka et al., 1998). This multipotent ability is recapitulated to a lesser extent *in vitro*, where upon differentiation most cells proceed to the giant cell type fate (Rossant & Tamura-Lis, 1981). However, syncytiotrophoblast derivatives have been reported (Hughes *et al.*, 2004). Several studies have already been carried out using TS cells as a model for placental development (Donnison *et al.*, 2005; Hughes *et al.*, 2004; Winger *et al.*, 2007; Wu *et al.*, 2003; Yan *et al.*, 2001) and first lineage allocation (Tanaka *et al.*, 2002).

1.3.1.2 Genes Involved in TS cells and Early Placental Development

There are over 100 genes known to be essential for correct placental development (Simmons & Cross, 2005), yet most of them are involved with morphogenesis and only a few are known to be important for trophoblast stem cell maintenance and differentiation (Papadaki *et al.*, 2007). *Cdx2*, *Eomes* and *Errβ* are required for early placental development, and now with using the TS cell model the precise functions of these genes in the extra-embryonic ectoderm is being elucidated.

The earliest known transcription factor specifically expressed by the trophoblast and required for TE cell fate specification is *Cdx2* (Strumpf *et al.*, 2005). *Cdx2* knockouts fail to implant suggesting a major defect in TE development. In addition in *Cdx2* knockouts the ICM marker *Oct4* fails to be restricted correctly; all cells of the blastocyst aberrantly express this ICM marker gene. Forced over-expression of *Cdx2* in embryonic stem (ES) cells promotes their differentiation into trophoblast like cells

(Niwa *et al.*, 2005; Tolkunova *et al.*, 2006) showing Cdx2 is sufficient to induce the trophectoderm lineage, however in the absence of *Oct4*, *Cdx2* is not required for TE specification (Niwa *et al.*, 2005). Instead *Cdx2* is required for the maintenance of mTS cell renewal. A forced reduction of Cdx2 in TS cells leads to a failure of self renewal and differentiation (Niwa *et al.*, 2005) indicating Cdx2 plays a role in maintaining the proliferative ability of the extra-embryonic ectoderm.

Eomesodermin (*Eomes)* is expressed in the early placental lineages (polar trophectoderm and extra-embryonic ectoderm) and knockouts die at around 6 days post conception (d.p.c.) (Russ *et al.*, 2000). *Eomes* mutants were shown to implant and are able to correctly specify the TE and ICM of the blastocyst; however, TS cells were unable to be derived indicating *Eomes* is also required for TS cell maintenance and hence extra-embryonic ectoderm maintenance. A third well known gene required for mTS cells and placental development is *Errβ* (Luo *et al.*, 1997). *Errβ* knockouts resulted in embryonic death by E9.5, with a failure to produce a chorion and an over production of giant cells. The synthetic inhibitor of Errβ, diethylstilbestrol (DES), disrupts the transcriptional activity of Errβ by preventing binding to its coactivator GRIP1 (Tremblay *et al.*, 2001). Treatment of mTS cells with DES results in differentiation to the giant cell fate even in the presence of Fgf4 and Activin. Additionally treatment of pregnant mice with DES early in pregnancy led to placental failure by E9.5. Placentas were characterised by an over abundance of giant cells. These results indicate *Errβ* plays an essential role in maintaining the stem cell population of the placenta. The studies on *Cdx2*, *Eomes* and *Errβ* show the close correlation between mTS cell expression/gene networks and the *in vivo* situation and the ability to use TS cells in determining the role of these genes in trophoblast cell renewal. Since these studies further genes have been discovered to play an important role in mTS cells. One gene recently found to be required in mTS cells is the Retinoblastoma (*Rb*) tumour suppressor gene. This gene was first identified as the gene responsible for human retinoblastoma cancers. Studies have shown that $Rb^{-/-}$ foetuses supplied with a wildtype placenta can survive to birth (Wu *et al.*, 2003). Targeted deletion of *Rb in vivo* resulted in an increase in the proliferation of extra-embryonic/chorionic trophoblasts (as determined by *Eomes* expression) and a global disruption of placental architecture, particularly the labyrinth and death of the embryos by E15.5. Cre mediated *Rb* ablation in TS cells had a similar phenotype to *Rb* knockouts conversely in differentiated cell types such as spongiotrophblast had no effect. Therefore *Rb* plays a critical role in

placental development through its role in TS cell function (Wenzel *et al.*, 2007). Another gene shown to be essential for mTS cells is *Sox2*. The primary defect in *Sox2* mutants occurs in the embryo proper, yet this is only partly rescued by using wild type ES to make chimeras. A secondary defect is observed to cause death through defects in development of the chorion. TS cells could not be derived from *Sox2* mutants confirming its essential role in maintaining the placental stem cell potential.

1.3.2 The Role of *Elf5* in TS Cell and Trophoblast Development

In 2005 two independent groups both produced a mutated non-functional *Elf5* allele using ES cells. Zhou *et al* (2005) created a targeting construct which interrupted exon 3 replacing it with a β-galactosidase construct, giving an Elf5 protein fused to β-galactosidase. Donnison *et al* (2005) created a targeting construct which interrupted exon 2 replacing it with a puromycin cassette. The heterozygous Elf5 $^{+/-}$ mice generated were crossed to produce homozygous Elf5$^{-/-}$ pups. However no homozygous pups could be generated indicating an essential role for Elf5 in embryonic survival (Donnison et al., 2005; Zhou *et al.*, 2005). *Elf5* heterozygous females showed impaired alveolar development and were not able to produce milk, indicating Elf5 is also essential for mammary gland development (Zhou et al 2005).

Elf5 belongs to the Ets family of transcription factors. It has been isolated from cDNA collected from adult mouse lung tissue using a probe for the Ets domain (Zhou *et al.*, 1998) and from differentiating keratinocytes (Oettgen *et al.*, 1999). Elf5 contains the conserved 85 amino acid Ets DNA binding domain along with a pointed domain responsible for protein-protein interactions. *Elf5* expression is restricted to epithelial tissue (mammary gland, lung, kidney, salivary gland, stomach, and early placental epithelia before embryonic day 13) and is therefore classed among the epithelial specific Ets factors (Zhou *et al.*, 1998).

The Ets family of transcription factors share a conserved DNA binding domain which binds to the core motif of GGA(A/T), found in the promoters and enhancers of various target genes (Sharrocks, 2001). DNA binding of Ets factors is usually caused by phosphorylation or interactions with a co-regulatory transcription factor. These protein-protein interactions between the other parts of an Ets protein determine its specificity; for example the pointed domain is found on a subset of Ets proteins and also found in receptor proteins and protein kinases which allow the formation of

homo or heterodimers (Sharrocks, 2001). Ets factors are commonly associated with tumourigenesis. For example the Ets-1 factor is an oncogene that can result in leukaemia (Sharrocks, 2001). More recently several Ets factors were found to be upregulated in human breast cancer cell lines including Elf5 (He *et al.*, 2007).

ETS factors also have important roles in development. For example Pointed P2 is essential for *Drosophilia* eye development (Treier *et al.*, 1995) and several Ets proteins play an important role in vasculogenesis and angiogenesis such as Fli-1 and Tel (Sharrocks, 2001)Apart from *Elf5*, several Ets factors have been found to be important for placental development such as *Ets2* (Georgiades & Rossant, 2006) and *Erf* (Papadaki et al., 2007).

Investigation into the lethal phenotype generated in homozygous null *Elf5* offspring phenotypes by Donnison *et al* (2005) showed that *Elf5* mutants died by E9.5. Using in situ hybridisation for marker genes of the extra-embryonic ectoderm, ectoplacental cone and the epiblast, revealed that *Elf5* mutants completely lacked the extra-embryonic ectoderm. The epiblast of the embryo was fused directly to the ectoplacental cone. Spatio-temporal analysis of *Elf5* expression showed that it was restricted to the extra-embryonic lineages; it was clearly detected at E5.5 to 8.5 in the extra-embryonic ectoderm and resulting chorion. Furthermore TS cells could not be derived from *Elf5* mutants and *Elf5* expression in wild type TS cells was reduced upon Fgf4 removal. *Elf5* has also been shown to be a target of Fgf4 signalling in mouse embryonic lung tissue (Metzger *et al.*).

Moreover, loss of the extra-embryonic ectoderm in *Elf5* mutants resulted in embryonic patterning defects. Correct Nodal signalling is required for formation of the anterior visceral endoderm (AVE). Correct Nodal signalling has been shown to be reliant on the extra-embryonic ectoderm (Beck et al., 2002; Guzman-Ayala et al., 2004). *Elf5* mutants correctly formed an AVE which appeared to migrate successfully to the future anterior of the embryo. However gastrulation did not proceed as evidenced by the absence of mesoderm tissue.

Taken together these results have shown *Elf5* to be essential for placental development through its role in maintenance of the trophoblast stem cell population residing in the extra-embryonic ectoderm and chorion. Secondly, the extra-embryonic ectoderm plays

an important role for gastrulation although it is not required for AVE formation (Donnison *et al*., 2005). The authors suggest a model whereby *Elf5* is a down stream gene of *Cdx2* and *Eomes* and is required for maintenance of extra-embryonic proliferation. The reasoning behind this was *Cdx2* was normally expressed in *Elf5* mutants up until E4.5 and polar trophectoderm was correctly specified; also the Elf5 homozygous mutant dies at a later stage compared to *Eomes* knockouts.

1.3.2.1 Other Ets Transcription Factors in Trophoblast Development

The related Ets gene *Ets2* has a very similar expression pattern and knock-out phenotype to *Elf5* (Georgiades & Rossant, 2006). *Ets2* expression is similar to Elf5 in that it is restricted to the extra-embryonic ectoderm. However following E 7.5 it is down regulated and unlike Elf5 it begins to be expressed in the primitive streak of the gastrulating embryo. *Ets2* knockouts lack an extra-embryonic ectoderm and are able to specify the AVE. Formation of mesoderm also did not occur supporting the role of the extra-embryonic ectoderm as essential for gastrulation (Georgiades & Rossant, 2006). However, in contrast to *Elf5* mutants, AVE migration to the anterior side was not completed correctly.

Finally, the Ets transcriptional repressor *Erf* has been implicated in extra-embryonic maintenance (Papadaki *et al*., 2007). Erk mutants die by E10.5 due to an expanded chorion and a lack in chorion differentiation. Additionally, TS cells were able to be derived and exhibited delayed differentiation as indicated by the retention of 10-20 fold higher expression of *Eomes*, *Errβ* and *Cdx2* compared to wild-type TS cells after Fgf4 withdrawal. Therefore, in contract to *Ets2* and *Elf5*, *Erf* expression is required for differentiation of the chorion and down-regulation maintains a stem cell state (Papadaki *et al*., 2007).

Clearly Ets transcription factors play important roles in maintenance and differentiation of the stem cell populations in the extra-embryonic ectoderm and resulting chorion. The defects in the extra-embryonic ectoderm have given interesting opportunities to allow a study of the extra-embryonic ectoderm's role in patterning through Nodal signalling and AVE formation and migration. However a detailed analysis of how Ets genes control maintenance or differentiation of the extra-embryonic ectoderm through the identification of their target genes has not been performed. Although Ets genes share the Ets DNA binding domain, they have also

been shown to have very distinct biological roles (Sharrocks, 2001), so it cannot be assumed that Ets2 and Elf5 have the same target genes, although the similarity in phenotypes suggests that they have overlapping functions.

1.3.3 The Purpose of this Study

The research carried out in this study focused on the role of *Elf5* in trophoblast stem cells as a model for the role of *Elf5* in maintaining the extra-embryonic ectoderm. By modulating the expression of *Elf5* using RNA interference (RNAi), it was hoped to ascertain the downstream target genes of Elf5 and shed some light on its pivotal role in TS cell maintenance. The change in expression levels of genes due to *Elf5* modulation were measured using a candidate gene approach and a global approach. Candidate gene analysis measured the change in expression of selected genes known to be crucial for placental cell type specification using real-time PCR. A global approach was employed using an oligonucleotide-based microarray to simultaneously measure the change in expression of thousands of transcripts due to changes of *Elf5* expression. Alongside Elf5's role in TS cells, the impact of loss of Fgf4 and Activin signalling was also investigated as gene expression responses to these pathways are also essential for TS cell maintenance.

Microarray analysis has been widely used to find the downstream targets of transcription factors in an attempt to identify gene regulatory networks in target cell lineages (Babaie *et al.*, 2007; Saito *et al.*, 2007; Tanaka et al., 2002; Wang *et al.*, 2007). Microarrays have the advantage of being able to screen transcriptional level changes on a genome wide scale, allowing the identification of novel target genes, rather than the small set of candidate genes tested by other methods. Microarrays are often used in conjunction with chromatin immunoprecipitation assays (CHIP on chip) to isolate (CHIP) and then identify (chip) a set of DNA transcripts which are bound by the transcription factor of interest. However, this method does not provide information on whether target genes are activated or repressed (Matoba *et al.*, 2006). By manipulating the expression of a transcription factor and then monitoring global expression, targets which are up or down regulated can be identified. A 24 hour time period following *Elf5* mitigation with siRNA was chosen to allow the identification of immediate down stream genes. A longer time period would have a greater chance of allowing secondary targets to also be changed. A similar approach combining RNAi with microarray

analysis has been previously used to identify down stream targets of the *Oct4* transcription factor (Babaie et al., 2007).

CHAPTER 2 :MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Mammalian Cell culture Reagents

2.1.1.1 Plasticware and consumables

TrypLE™ Express, trypsin replacement enzyme (Gibco, Invitrogen life technologies Auckland, NZ)
Multidish 6 wells (Nunc, Kamstrupvej, Denmark)
Multidish 12 wells (Nunc, Kamstrupvej, Denmark)
Tissue Culture dish 60x15 (Nunc, Kamstrupvej, Denmark)
Tissue Culture dish 100x20 (Nunc, Kamstrupvej, Denmark)
Cryovials (Nunc, Kamstrupvej, Denmark)
Crystal Violet (BDH Laboratory Supplies, England)
Dimethyl Sulphoxide Hybri Max (DMSO) (Sigma-Aldrich, Ayrshire, UK)

2.1.1.2 Cell Culture Media

All cell culture media was stored at 4°C for no longer than 6 weeks. The protocols for all medias were obtained from Placenta and Trophoblast methods, volume 1.(Quinn *et al.*, 2006)

2x RPMI Medium 1640

1 packet of RPMI Medium 1640 powder (Gibco, Invitrogen life technologies, Auckland, NZ)
500ml MQ H2O
2.0g NaHCO3 (Cell culture grade, Sigma-Aldrich, USA)
pH adjusted with 10M HCl
Filter Sterilised (0.22μm filter)

2x DMEM F12

1 packet of DMEM F12 medium powder (Gibco, Invitrogen life technologies, Auckland, NZ)
500ml MQ H2O
2.438g NaHCO3 (Cell culture grade, Sigma-Aldrich, USA)

pH adjusted with 10M HCl

Filter Sterilised (0.22µm filter)

Mouse trophoblast stem cell media (mTS media)

1x RPMI 1640 (2x as described above)

20% fetal bovine serum (FBS) (ICP biologicals, Auckland, NZ)

1mM Sodium Pyruvate (100x Gibco, Invitrogen life technologies, Auckland NZ)

0.1mM βMercaptoethanol (Sigma-Aldrich, USA)

2mM L-Glutamine (200mM, 100x, Invitrogen life technologies, NZ)

1x Antibiotic/Antimycotic (100x::10,000units of penicillin, 10,000µg of streptomycin, 25µg amphotercin B per ml) (Invitrogen life technologies, NZ)

Reverse Osmosis mQ H2O (autoclaved to sterilise, Millipore Corporation, USA)

Mouse embryonic fibroblast media (mEF media)

1x DMEM F12 media (2x as described above)

10% FBS

1mM Sodium Pyruvate

1x Antibiotic/Antimycotic

0.05mM βMercaptoethanol (Sigma-Aldrich, USA)

Reverse Osmosis mQ H2O (autoclaved, 121°C 15min)

70% Conditioned media + Fgf4 and Heparin (70%CM + F4H)

70% mEF Conditioned Medium (2.2.1.3)

30% mTS media

1xFgf4

1x Heparin

2x mouse trophoblast stem cell freezing media

50% FBS

30% mTS media

20% DMSO (Sigma-Aldrich, UK)

Cool to 4˚C

2x mouse embryonic fibroblast cell freezing media

50% FBS

30% mEF media

20% DMSO

Cool to 4˚C

2.1.1.3 Growth Factors and Antibiotics

Fibroblast growth factor-4 human recombinant, 25μg (Sigma-Aldrich, USA)

Resuspend in 1ml PBS (Oxoid, UK) 0.1%Bovine Serum Albumin (Invitrogen life technologies, Aucklnad, NZ) w/v. This gives a 1000x solution.

Make 50μl aliquots and store at -80°C

Defrost as required and keep at 4°C

Heparin 10,000 units (Sigma-Aldrich, USA)

Resuspend in PBS to give a 1000x stock concentration of 1mg/ml

Aliquot as 1ml lots and store at -80°C

Thaw as required and store at 4°C

Mitomycin C 2mg (Sigma-Aldrich, USA)

Resuspend in 2ml of PBS

Aliquot and store at -80°C

Puromycin (Sigma-Aldrich, USA)

Dissolve in mQ H2O 1mg/ml

Filter sterilized 0.22μm syringe filter

Doxycycline Hydrate (Sigma-Aldrich, USA)

Dissolve in mQ H2O at a concentration 1mg/ml to give 1000x solution

Filter sterilize through 0.22μm filter (Pall Corporation, Michigan, USA)

Store at -20°C in the dark for up to one year

Geneticin (G418 Sulfate) (Gibco, Invitrogen life technologies, NZ)

Dissolve 1g in 70ml 1x RPMI to make a 10mg/ml stock

2.1.1.4 Transfection Reagents

Lipofectamine™ 2000 stored at 4°C (Invitrogen life technologies Auckland, NZ)

1x Optimem reduced serum medium (Gibco, Invitrogen life technologies, Auckland NZ)

Reduced serum mouse trophoblast medium for transfections

1x RPMI 1640 (2x as described above)

5% FBS

1mM Sodium Pyruvate

0.1mM βMercaptoethanol

2mM L-Glutamine

Sterile Reverse Osmosis mQ H_2O

2.1.1.5 Transfected RNAi/DNA constructs:

All RNAi molecules were stored at -80°C and all DNA constructs were stored at -20°C.

Stealth RNAi dsRNA x4, diluted to a working concentration of 20pmol/µl in RNAse free water (Invitrogen life technologies)

Stealth RNAi Negative Control Duplexes, medium GC duplex 20pmol/µl stored at -80°C (Invitrogen life technologies, Australia)

Block-iT Fluorescent Oligo (Invitogen Corporation)

pTracer-Elf5 DNA construct (supplied by B.Brophy, using pTracer™ –CMV2 plasmid backbone(Invitrogen life technologies))

pTet-On gene expression plasmids which includes pTet-On, pTre2hygLuc and pTRE2hyg (BD Biosciences-Clonetech, California, USA)

pGL71 puromycin resistance vector fragment (kindly supplied by Craig Smith)

PCH110 β-Galactosidase construct (kindly supplied by J.Bracegirdle)

pRL Renilla Luciferase Vector (kindly supplied by J.Bracegirdle)

pGL3P Firefly Luciferase positive control vector (kindly supplied by J.Bracegirdle)

2.1.2 Biochemical analysis reagents

All kit components were stored under the manufacturer's instructions. In general this was at -20°C except for the luciferase substrate in both the luciferase and dual luciferase kits. Once reconstituted this was stored at -80°C.

Luciferase Assay System (Promega, WI, USA).

Dual-Luciferase Reporter assay system (Promega, Madison, USA)

B-Galactosidase Enzyme Assay System (Promega, Madison, USA)

96-well flat bottomed polystyrene plate (Nunc, Kamstrupvej, Denmark)

Parafilm (Pechiney plastic packaging, USA)

2.1.3 Molecular Biology Reagents

2.1.3.1 General Reagents

TRIZOL Reagent (Invitrogen life technologies)

RNase Away (Molecular Bioproducts Inc., USA)

LightCycler 480 SYBER Green 1 Master (Roche Diagnostics, Manheim, Germany)

Applied Biosystems SYBER Green PCR Master Mix (Applied Biosystems, Victoria, Australia)

Applied Biosystems 96 well plate (Applied Biosystems, Victoria, Australia)

Oligo $dT_{12\text{-}18}$ 500µg/µl (Invitrogen life technologies)

Glycogen 10mg/ml (Roche Diagnostics, Germany)

DTT (Invitrogen life technologies, NZ)

dNTPs 10mM (Roche, Germany)

ABgene 1kb DNA ladder (ABgene, Surrey, UK)

ABgene 100bp DNA ladder (ABgene, Surrey, UK)

ReddyRun gel loading buffer (ABgene, Surrey, UK)

Ultrapure™ Agarose powder (invitrogen life technologies)

Ethidium Bromide 10mg/ml (Sigma-Aldrich, USA)

DEPC treated water

    0.01% diethyl pyrocarbonate (DEPC; Sigma-Aldrich, USA)

    mQ water

    Left mixing overnight at room temperature

    Autoclaved

3M sodium acetate (BDH, England)

    DEPC treated water

    Adjusted to pH 5 with 3M acetic acid (J.T. Baker, USA)

    Filter sterilized with a 0.22µm acrodisc syring filter (Pall Corporation, USA)

DNA purification Maxi Kit (QIAGEN Incorporated, BioLab Scientific Limited, NZ)

Wizard gel clean up (Promega, Madison , USA)

Geneclean II kit (QBiogene, USA)

LB/Amp broth

    25g Luria Broth powder (Invitrogen life technologies)

    Dissolved in 1L mQ water

    pH 7, adjusted with 10M NaOH

    Autoclaved, stored at 4°C

    Just prior to use, add ampicillin to give a final concentration of 100µg/ml.

LB + Ampicillin plates

    25g Luria Broth powder (Invitrogen life technologies)

    1.5g agarose

    Dissolved in 1L mQ water

    pH 7, adjusted with 10M NaOH

Autoclaved, allowed to cool

When approximately 50°C add filter sterilised ampicillin (Sigma- Aldrich), to give a final concentration of 100μg/ml, pour into bacterial culture dishes and when set store at 4°C

DH5α™ competent EColi cells (Invitrogen life technologies)

QIAGEN Plasmid Maxi Kit (QIAGEN, USA)

2.1.3.2 Buffers

TE Buffer

10mM Tris pH 8.0 with HCl (BDH, England)

1mM EDTA

DEPC treated water

Autoclaved

50x TAE buffer

2M Tris pH 8.0

0.57% acetic acid (J.T. Baker, USA)

500mM EDTA

mQ water

Autoclaved

Proteinase K Lysis Buffer

100mM NaCl

10mM Tris pH 8.0 with HCl (BDH, England)

25mM EDTA

0.5% SDS

1mg/ml Proteinase K(Roche, Germany) added fresh

2.1.3.2.1 Extraction/lysis Buffers used in miniprep plasmid preparation:

Made using the protocol given in Molecular Cloning, appendix 1 (Sambrook & Russel, 2001a), all made solutions were stored at 4°C.

Alkaline Lysis Solution I

50 mM glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Autocalved

Alkaline lysis solution II (made up fresh each time)

0.2 M NaOH

1% (w/v) SDS

Alkaline Lysis Solution III

3 M Potassium acetate

5 M glacial acetic acid

mQ water

2.1.3.3 Enzymes

Deoxyribonuclease 1, Amplification Grade (Invitrogen life technologies, NZ)

SuperScript™ III Reverse Transcriptase (Invitrogen life technologies, NZ)

RNAseOUT ™ Ribonuclease Inhibitor (Invitrogen life technologies, NZ)

Calf Intestinal Phosphatase (Roche, Indianapolis, USA)

Xho1 Restriction Enzyme (Roche, Indianapolis, USA)

BamH1 Restriction Enzyme (Roche, Indianapolis, USA)

Apa1 Restriction Enzyme (Roche, Indianapolis, USA)

EcoRV Restriction Enzyme (Roche, Indianapolis, USA)

Klenow Enzyme, DNA Polymerase I large fragment (Roche, USA)

T4 DNA Ligase (Invitrogen life technologies)

FastStart Taq DNA polymerase (Roche, Germany)

Proteinase K 20mg/ml (Roche, Germany)

2.1.3.4 Solvents

Ethanol-absolute (analytical grade) (BioLab Australia Ltd)

Propan-2-ol (analytical grade) (BioLab Australia Ltd)

Chloroform (MERCK, Darmstadl, Germany)

Phenol:Chloroform:Isoamyl alcohol (Invitrogen life technologies, NZ)

2.1.3.5 Microarray Reagents

GeneChip® Eukaryotic Poly-A RNA Control Kit (Affymetrix Inc, California, USA)

GeneChip® One-Cycle cDNA Synthesis Kit (Affymetrix Inc, California, USA)

GeneChip® Sample Cleanup Module (Affymetrix Inc, California, USA)

GeneChip® IVT Labelling Kit (Affymetrix Inc, California, USA)

GeneChip® Hybridisation Control Kit (Affymetrix Inc, California, USA)

12x MES Stock Buffer

64.61g MES Hydrate (cat# M5287, Sigma-Aldrich)

193.3 MES Sodium Salt (cat# M5057, Sigma-Aldrich)

800ml mQ filtered H_2O

Mix, adjust to pH 6.5-6.7 with 10M HCL, Filter with 0.2μm filter

2x Hybridisation Buffer (25mls)

4.15mls 12x MES stock buffer

8.85mls 5M NaCl (analytical grade) (BioLab scientific Ltd, Australia), dissolved in DEPC H_2O and autoclaved to sterilise

2.0mls 0.5M EDTA Disodium salt (Gibco ultrapure, Invitrogen life technologies)

0.05ml 10% Tween 20 (BioRad Laboratories, C.A., USA)

9.95mls DEPC H_2O (Supplied by Molecular biology laboratory)

Store at 2-8°C away from light

Herring Sperm 10mg/ml (Promega Corporation, USA)

Bovine Serum Albumin 50mg/ml (Invitrogen life technologies)

DMSO (>99.9%) (Sigma-Aldrich)

18x GeneChip® Mouse Genome 430 2.0 arrays (Affymetrix Inc, California, USA)

2.1.3.6 Real-Time PCR primers

Primers were dissolved in TE buffer to make 1000μM stock solution and stored at -80°C. A working solution was made by diluting to 10μM in TE and these were stored at -20°C. Primers were purchased from Sigma GeneSys (Sigma-Aldrich, NSW, Australia) or from Invitrogen (Invitrogen life technologies, Auckland, New Zealand).

2.2 METHODS

All Experimental work was carried out at AgResearch's Ruakura Campus, Hamilton, New Zealand, except for hybridization and scanning of the microarrays which was carried out by Liam Williams at Auckland University's Affymetrix facility.

2.2.1 Cell Culture Protocols

All cell culture work was carried out aseptically in a laminar flow cabinet (Westinghouse Pty Ltd, NSW, Australia) containing a 0.2μm HEPA (high efficiency particulate air) filter, which blows sterile air outwards. All surfaces and media bottles were sprayed with 70% ethanol before beginning. Cells were grown under 5% CO_2 in a water jacketed incubator (Forma Scientific) and the pH of the culture media was monitored by the colour of phenol red in the media. All medium added to the cells were pH adjusted to approximately 7.2-7.4 using sterile 1M HCl (as judged by the phenol red colour) and warmed to at least room temperature before use to prevent shocking the cells. All protocols regarding mTS and mEF cell culture except for cell counting and transfection are based on those taken from the book 'Placenta and Trophoblast, methods and protocols, volume 1, chapter 11 (Quinn et al., 2006).

2.2.1.1 Mouse Trophoblast Stem Cell Growth and Maintenance

Mouse trophoblast stem cells (mTS cells) were provided by James Cross' laboratory, University of Calgary Faculty of Medicine, Canada. mTS cells are maintained in 70% fibroblast conditioned mTS medium supplemented with Fgf4 and Heparin (F4H) (2.1.1.2). All of these factors must be provided to prevent precocious differentiation of the mTS cells. The 70% conditioned medium + F4H was changed every second day to maintain a suitable concentration of growth factors. Cells were subcultured at approximately 80% confluence to prevent contact inhibition of cells which may also result in differentiation and normally split 1:4, as splitting at a too high dilution can also result in differentiation (Quinn et al., 2006). MTS cells were also routinely cryopreserved and weren't cultured when not needed to keep the passage number of the cells low (the cells from J.Cross were given a passage number of 0).

2.2.1.2 Mouse Embryonic Fibroblast Growth and Maintenance

Mouse embryonic fibroblasts (mEFs) were supplied by D. Pearton and P. Pfeffer using day 13-14 mouse embryos. After the primary mEFS were established they were split 1:6, grown to confluencey and then frozen and stored in liquid nitrogen (2.2.1.6). MEFs were grown in mEF media (2.1.1.2) and were grown to provide conditioned media for mTS cell maintenance. The passage number of mEFs was kept low (less than 5) to maintain their growth enhancing activity (Nagy *et al.*, 2003). MEFs were grown in 100mm tissue culture plates, changing the media every two to three days. Once they reached confluency they were subcultured 1:5 or treated with mitomycin C.

2.2.1.3 Mitomycin C treatment of mEFs and preparation of Conditioned Media

MTS cells can either be grown on a feeder layer of mEFs or grown in 70% mEF conditioned mTS medium. MTS cells were grown in 70% conditioned medium so that there was no problem of ensuring the feeder layer was also resistant to the antibiotics used during antibiotic selection. Confluent mouse embryonic fibroblasts (mEFs) were treated with mitomycin C at a concentration of 10μg/μl for 4 hours at 37°C by adding 100μl of a 1mg/ml solution of mitomycin C to 100mm plates of mEFs containing 10mls of mEF media. MEFs were then rinsed twice with PBS. The PBS was aspirated and the mEFs were dissociated with TrypLE Express at 37°C for 5-10min. The treated mEFs were then pipetted into a centrifuge tube and centrifuged at 112 x *g* for 5min. The supernatant was aspirated and the cell pellet resuspended in 2ml of mTS media. An aliquot of 50μl was taken for counting using a haemocytometer (2.2.1.5). $2x10^6$ mEFs plated were plated into a 100mm tissue culture dish and 11ml of mTS media added. Seventy-two hours later the mTS media was collected and replaced. Collected mTS media was centrifuged at 112 x *g* for 5min to remove cell debris and filtered using 0.2μm Serum Acrodiscs (Pall Corporation, MI, USA). This was stored at -20°C for long term. Short term storage (up to 2 weeks) was at 4°C.

2.2.1.4 Subculturing of Mammalian Cells

Cells were rinsed with PBS then a small amount of TrypLE was applied (just enough to cover the bottom of the dish). Cells were placed in a 37°C CO_2 incubator for 5 minutes and then checked every couple of minutes under a 40*x* inverted phase contrast microscope (Olympus) for rounding up of the cells and their loosening from the bottom of the dish. An equal volume of mTS medium was then added and the cells

were then pipetted into a centrifuge tube, while rinsing the bottom of the dish to collect all cells. This was spun at 110 x *g* for 5 minutes in a Mistral 1000 benchtop centrifuge (Labsupply, Auckland, New Zealand). The supernatant was then aspirated and the cells resuspended in 2mls of growth media. Cells were then either counted if a specific plating density was required or subcultured to continue growing.

2.2.1.5 Counting of Mammalian Cells

A total cell count was obtained by removing 50μl of a cell suspension and mixing with 450μl of a 0.04% crystal violet solution in 0.5 M citric acid to stain the cell nulcei. This was warmed for a few minutes at 37°C and vortexed. A coverslip was placed on the ridges of a haemocytometer chamber and then some of the cell sample was pipetted on to the edge of the coverslip. The sample was drawn up by capillary action and then the haemocytometer was inspected using a 100x microscope. Four large squares on the haemocytometer were counted for cell nuclei. The cell count was given by:
Cell count (cells/ml) = (number of cells counted/4) x 10(dilution factor) x 10^4

2.2.1.6 Cryopreservation and Thawing of Cells

Cells were frozen at a final concentration of 1-2 x 10^6 cells/ml. After a cell count to ensure adequate numbers of cells, an equal volume of freshly made 2x solution of cell freezing media at 4°C was added (2.1.1.2). This contains DMSO which protects over dehydration of the cells during the freezing process. The cells suspension was pipetted into 1ml cryovials; the vials were then clearly labelled and placed in a NALGENE™ cryo 1°C freezing container filled with isopropanol which was placed in a -80°C freezer overnight. This allows slow freezing of the cells to prevent ice crystals forming intracellularly. The next day the vials were shifted to long term storage in liquid nitrogen.
Thawing:
Cells were rapidly thawed by placing vials in a 37°C water bath for 1-2minutes. The contents of the cryovial were then transferred to a centrifuge tube containing 5ml of fresh medium to dilute the DMSO slowly and spun at 100 x *g* for 5 minutes. The supernatant containing the DMSO was removed as this is toxic to cells and the cells were resuspended in normal culture media and plated. The media was replaced the following day.

2.2.1.7 Transfection of mTS Cells

All mTS cell transfections were carried out using lipofectamine 2000 reagent (Invitrogen Corporation). Lipofectamine 2000 is a cationic lipid solution which works by interacting with the negative charges of DNA or RNA condensing these macromolecules into compact structures. It also forms a liposome structure in water and because of its cationic charge and lipophilic nature it interacts with the negatively charged hydrophobic membranes of cells (Ausubel *et al.*, 1999). Lipofectamine 2000 was mixed gently before use by inverting the tube a few times. DNA constructs or siRNAs were defrosted and kept on ice, they were mixed by flicking the tube a few times and then collecting the contents at the bottom of the tube by a minicentrifuge. DNA was not vortexed to avoid shearing. Protocols for siRNA and DNA transfections are all based on the manufacturer's protocol (Invitrogen life technologies, 2006). Four types of transfections were carried out:

i. Stealth siRNA transfection for knockdown of *Elf5* mRNA
ii. Transient transfection of pTracer-Elf5 for *Elf5* over-expression
iii. Stable transfection of the pTetOn construct to develop an mTS cell line expressing the rtTA gene
iv. Transient cotransfections of 'mTS-pTetOn' cells with pTre2hyg-Luc and a normalizing construct for use in screening rtTA gene expression and activation.

2.2.1.7.1 Seeding the wells:

In all cases the general protocol was to seed a 6 well or 12 well plate with mTS cells the day before transfection, this ensured cells were healthy and rapidly growing at the time of transfection, enhancing transfection efficiency (Ausubel et al., 1999). MTS cells were rinsed with PBS and then disassociated using TrypLE for 5-10 minutes at 37°C whilst monitoring progress by checking the cells with an inverted phase contrast microscope set to x40 magnification. TrypLE treatment for too long causes differentiated cell types such as giant cells which are much more resistant to disassociation to also become loose. TrypLE was neutralized by adding an equal volume of mTS media, then the dish was shaken to loosen cells. The cell suspension was pipetted into a centrifuge tube whilst washing loose cells off the bottom of the dish and spun for 3mins at 112 x *g* using a Mistral 1000 benchtop centrifuge (Labsupply, Auckland, New Zealand). The supernatant was aspirated and then the cells were resuspended in 2-3mls of 70% CM + F4H and a cell count was performed (2.2.1.5). The optimum seeding density was

determined previously experimentally in order to obtain the confluency required for each type of transfection. Cells were allowed to grow overnight in 70%CM + F4H.

2.2.1.7.2 General Transfection Procedure

The DNA/siRNA was diluted into Optimem reduced serum medium (Gibco) in a polypropylene tube or small glass bottle. Lipofectamine 2000 was then diluted into a separate tube containing an equal volume of Optimem and mixed gently. After 5 minutes incubation at room temperature the Lipofectamine 2000 and DNA/siRNA solutions were mixed and left at room temperature for 20 minutes to form complexes. Optimem reduced serum media was used because it is recommended by the manufacturers; serum proteins in media can compete with DNA/siRNA for binding with cationic lipids (Ausubel *et al.*, 1999).

During the 20min incubation the 70%CM + F4H was removed from the wells to be transfected and replaced with enough reduced serum/ no antibiotics mTS media (2.1.1.4) to give a total volume of 2mls for a $10cm^2$ well or 1ml for a $4cm^2$ well once the complex mixture was added. The presence of antibiotics can kill the cells during a transfection (Invitrogen life technologies, 2006).

After the 20minute incubation the siRNA/DNA/lipofectamine 2000 complex was added to each well drop-wise, and then mixed in by gently rocking the plate back and forward. 4-5 hours later the media was replaced with 70%CM + F4H to increase the serum and the growth factors for the cells. According to the manufacturers 4-6hours is adequate time for the transfection to occur. 24 or 48 hours after the commencement of the transfection the cells were harvested and their mRNA extracted for analysis of gene expression (2.2.3.2.1) or cell lysate collected for biochemical analysis (2.2.2)

2.2.1.7.3 Transfection of mTS Cells with siRNA

Stealth siRNAs were designed using an Invitrogen program available over the internet (Invitrogen life technologies, 2004a) using the mouse mRNA sequence for the Elf5 gene (NCBI accession number NM-010125). Four proposed siRNAs were designed and tested for efficacy in mTS cells.

Cells were seeded into wells at a density of $2x10^4$ cells/cm^2 for a 24 hour long transfection or $1.5x10^4$cells/cm^2 for a 48 hour long transfection. This enabled cells to

be at 30-40% confluency at the time of transfection and also that cells did not overgrow by the time of harvesting. Each transfection also included at least one control well which was transfected with the siRNA negative control oligo. This provided a control for sequence independent RNAi effects (Invitrogen Life Technologies, , 2004b) and was transfected in an identical manner to the *Elf5* target siRNAs. Transfection was generally carried out in a 6 well plate ($10cm^2$) well using 100pmol of siRNA as this seemed to give the best knockdown efficiency.

The stealth siRNA was diluted by mixing 5µl of 20pmol/µl siRNA in 250µl of Optimem per well (this gives 100pmol stealth siRNA). 5µl of Lipofectamine 2000 was diluted in 250µl of Optimem per well.

2.2.1.7.4 Transient Transfection with the pTracer-Elf5 Construct

On the day before the transfection, cells were seeded as mentioned above (2.2.1.7.1) at a density of $4x10^5$cells/ $10cm^2$ well to obtain a confluency of 70-80% at transfection. The general transfection procedure given in 2.2.1.7.2 was followed. At least one control well was included for each experiment; which was not transfected and used to check basal *Elf5* expression. 4µg of circular pTracer-Elf5 DNA was transfected by diluting in 250µl Optimem for each well. 10µl of Lipofectamine was added to 250µl Optimem per well. 24 hours after transfection the cells were harvested in Trizol for RNA extraction (2.2.3.2.1).

2.2.1.7.5 Stable Transfection

In order to develop an mTS cell line expressing the rtTA protein for the inducible expression of *Elf5* (chapter 4) mTS cells were co-transfected with a puromycin resistance gene under the control of the mouse phosphoglycerine Kinase (PGK) promoter, a 'strong housekeeper' (Sun & Storb, 2001), and linearised pTetOn plasmid. A co-transfection had to be done because the mTS cells used were already resistant to G418, the antibiotic resistance gene carried on the pTetOn construct (section 4.2.1.1). This was transfected at a molar ratio of 10 pTetOn: 1puromycin to ensure any puromycin resistant mTS cells would have a high chance of also carrying the pTetOn construct. On the day before the transfection cells were seeded as mentioned above (2.2.1.7.1) at a density of 4x10^5cells/ $10cm^2$ well to obtain a confluency of 70-80% at transfection. The general transfection procedure given in 2.2.1.7.2 was followed. The total amount of DNA transfected was 4µg diluted in 250µl of Optimem per well. 10µl

of Lipofectamine 2000 also diluted in 250µl of Optimem was also used. The amount of each construct was calculated as follows to achieve a 10:1 molar ratio in a total of 4µg DNA:

pTetOn=7.4kb Puromycin=6kb,

	pTetOn	Puromycin
Plasmid size	7.4kb	6kb
Wanted molar ratio	10	1
Ratio	(7.4/6) x 20 x 1µg	1µg
Amount needed for	24.7µg x 4µg	1µg
a total of 4µg	24.7µg + 1µg	25.7µg
	=3.8µg	=0.2µg

The control for DNA transfection was a well which was not transfected. During antibiotic selection cells from this well should all eventually die, enabling the time for antibiotic selection to be judged. 24hours post transfection the cells were subcultured (2.2.1.4); each well of cells plated into 2x 10cm dishes (total surface area 120cm^2) the seeding density for antibiotic selection was found during the pilot experiments (section 4.2.1.3). This was to give a low cell density for selection so there is a small chance of selecting a colony which originated from two separate transfection events. The following day the media was replaced with fresh growth media containing the antibiotic for selection. In the case of puromycin this was added at an optimised concentration of 1µg/ml (4.2.1.2). The media was changed every second day for fresh 70%CM + F4H + puromycin until all negative control cells were dead and well separated colonies of about 0.5cm-1cm in diameter could be seen. Colonies were then 'picked' (2.2.1.8)

2.2.1.7.6 Transient Transfection for Reporter Assay Screening

To establish the Tet-On inducible expression system in mTS cells the pTet-On vector which carries the rtTA gene was co-transfected with a puromycin resistance vector (2.2.1.7.5). Cells were then plated at a low density and subjected to antibiotic selection and then colonies of antibiotic resistant clones were picked and grown individually (2.2.1.8). These 'mTS-pTetOn' cell lines were then screened for expression of the rtTa protein. On addition of doxycycline, cells expressing rtTA should be able to induce expression of a reporter gene carrying the TRE (Tetracycline response element). A Luciferase reporter vector (pTRE2Hyg-Luc) was transiently transfected into the 'mTS-

pTetOn' cells to screen for cells with the greatest expression of rtTA. To control for variation in transfection efficiency and cell number pTRE2Hyg-Luc was always cotransfected with another vector, either PCH110 which expresses β-Galactosidase or pRl which expresses *Renilla* (*Renilla reniformis*) luciferase. Two 4cm^2 wells for each line were seeded at a density of 8x10^{4}cells/4cm^2, to obtain a confluency of 40-50% at transfection. Although a higher confluency is recommended at transfection for DNA transfections the 'mTS-pTetOn' cells were not harvested until 48hours after transfection to give adequate time for the expression of the transgene proteins; the maximum confluency at harvesting was suggested to be 95% in order to obtain efficient lysing of the cells.

A total of 1.6μg of DNA was transfected per well. For co-transfections a mass ratio of 1:1 was used. Each transfection included four wells of mTS cells that had not been previously transfected with pTetOn to act as controls:

i. A positive control, which was cotransfected with 0.8μg of pGL3P (5kb), this expresses firefly luciferase under control of an SV40 promoter and 0.8μg of the normalizing vector (PCH110 [7.1kb] or pRL [4.1kb])
ii. Negative control; 'mock transfected' to give basal measurements
iii. Inducible positive control; transfected with 0.53μg each of pTetOn (7.4kb), pTre2hyg-Luc (7kb) and normalizing vector. After transfection doxycycline is added. Gives maximum expected induced luciferase expression.
iv. Inducible negative control; as in inducible positive control above however with no doxycycline added. Gives uninduced background expression.

The general transfection procedure was followed (2.2.1.7.2). 1.6μg of DNA was diluted in 100μl of Optimem for each well. 4μl of Lipofectamine 2000 was diluted into the same volume of Optimem for each well. After the transfection the media was changed for 70%CM +F4H, to one well of each mTS-pTetOn cell line was added 1μl of 1mg/ml doxycycline. Doxycycline was also added to all of the control wells except the inducible negative well. 48hours after the transfection the cell lysates were assayed (2.2.2).

2.2.1.8 Picking Stable mTS Cell Lines

This protocol is based on one accessed on the Corning website (Ryan, 2005).Using an inverted phase contrast microscope (Olympus) set to x 4 magnification circles were

drawn around each colony on the bottom of the dish. These were then 'picked': growth media was aspirated and the dish was rinsed in PBS. This was aspirated and then using sterile forceps 'cloning rings' (made by cutting the wide 0.5cm end of a pipette tip, and then autoclaving to sterilise) were dipped in sterile silicon grease. A cloning ring was placed firmly over a colony to create a seal and then 0.2ml of TrypLE was added to the cylinder. The dish was incubated for 5 minutes at 37°C and then checked to see cells had started to round up and come off the bottom of the dish. A few drops of mTS media were added and cells were aspirated using a pipette and plated into a well of a 12 well plate in 70%CM + F4H. The next day the media was changed and puromycin 1μg/ml added to maintain the selective pressure.

2.2.1.9 Screening of mTS Cells for Insertion of Foreign DNA

Isolated antibiotic resistant mTS cell lines were screened for a transgene by firstly extracting their DNA, running a PCR reaction using primers for the transgene (section 2.2.2.9) and finally running about 1/5th of the PCR reaction on an agarose gel containing ethidium bromide to check for the presence of the target DNA band (section 2.2.2.6).

2.2.1.9.1 Extraction of Genomic DNA from mTS Cells

Cells around 60-90% confluent in a 10cm^2 well were rinsed with PBS (Oxoid) and treated with TRYple (Invitrogen) until they had lifted. They were then transferred to a 0.65ml microcentrifuge tube and centrifuged for 10minutes, 1400rpm at 4°C (Eppindorf centrifuge 541D, Germany). The supernatant was aspirated and the pellet resuspended in 5μl of proteinase K lysis buffer which contained freshly added proteinase K (Roche, Germany) at a final concentration of 1mg/ml. The cell samples were heated at 55°C for 1 hour. The contents of each tube were collected at the bottom by briefly centrifuging and then 45μl of PCR grade water (Roche, Germany) was added to each sample and mixed by pipetting. 0.5μl was then used in a PCR reaction.

2.2.1.10 Media changes

mTS cells are known to require two growth factors; Fgf4 and Nodal. Removal of either of these results in the TS cells beginning to differentiate and no longer be pluripotent. To determine the underlying molecular changes that occur in this process mTS cells

were grown in mTS medium containing different combinations of growth factors as detailed in Table 1:2.2-1.

The cells were seeded as described in 2.2.1.7.1 except at a density of $4x10^5$ cells/well in a $10cm^2$ well. The following day the normal growth media was exchanged for one of the four types mentioned in Table 1:2.2-1. The cells were grown in this media for 24 hours and then harvested for RNA extraction (2.2.3.2.1).

Table 1:2.2-1 Media combinations for mTS cell experiments

Media Type	Nodal/Activin	FgF4
Normal mTS cell growth media (70%CM + F4H)	Yes	Yes
mTS media only	No	No
mTS media + recombinant Activin	Yes	No
mTS media + FgF4	No	Yes

2.2.1.11 Flourescent Microscopy

As a means of analyzing transfection efficiencies the mTS cells were transfected with DNA or siRNA constructs which express flurochromes that could be visualized under fluorescent microscopy. Cells could be visualized in their growing state (in tissue culture dishes) or after being fixed on slides and stained with a fluorescent dye such as Hoest which marks DNA and hence cell nuclei. A Leitz DMIRB fluorescent microscope was used (Leica, Germany).

2.2.1.12 Flow Cytometry Analysis of DNA content

Approximately $1x10^6$ cells were harvested using Tryple (Invitrogen Life Technologies) to disassociate cells from culture dishes. Cells were then spun at 1000rpm for 5 minutes in a Mistral 1000 bench top centrifuge (Labsupply, Auckland, New Zealand). The supernatant was discarded and cells resuspended in 70% fridge cold ethanol (BioLab Australia Ltd). Cells were fixed in ethanol for a minimum of 30 minutes before centrifuging the cells again at 1000 rpm for 5 minutes. The supernatant was discarded and cells resuspended in 1ml of PBS (Oxoid, UK). This washing step was

repeated twice. 100µl of DNAse free ribonuclease (100µg/ml; Sigma) was added and left at room temperature for 5 minutes. Finally 400µl of propidium iodide was added (50µg/ml) to stain the DNA. Flow cytometry analysis was performed on the samples by Sue Beaumont at 488nm.

2.2.2 Biochemical Analysis Protocols

Biochemical analysis was performed on 'mTS-pTetOn' cells to assay for the ability of each cell line to inducibly express the transiently transfected firefly (*Photinus Pyralis*) luciferase gene under the control of the TRE in the presence of doxycycline (for further background see Chapter 4). To control for variable transfection efficiency and cell number pTRE2hyg-Luc was cotransfected with either a β-Galactosidase expressing DNA construct (PCH110) or a *Renilla* (*Renilla reniformis*) luciferase expressing construct (pRL) (2.2.1.7.6) and the expression of the reporter firefly luciferase normalized to either β-galactosidase or *Renilla* luciferase measurements. Three different assays were performed: Firefly luciferase assay, βGalactosidase assay and a dual luciferase assay which measured both types of luciferase in one assay.

2.2.2.1 Luciferase Assay System

The Luciferase Assay System kit (Promega, USA) was used to measure firefly luciferase expression.The firefly luciferase enzyme produces light by oxidizing luciferin in the prescence of ATP. The protocol for this assay was performed with a modified version of the protocol by Promega. Lysis of the cells was by reporter lysis buffer from the β-galactosidase enzyme assay (Promega, USA) and the protocol for lysis with this buffer was followed from the β-galactosidase enzyme assay protocol (protocol available at www.promega.com/tbs/tb097/tb097.pdf) this is because the lysis reagent supplied in the luciferase assay kit is incompatible with the β-Galactosidase assay. The volume of cell lysate and luciferase assay buffer was halved in the assay following advice from J. Bracegirdle who routinely performs this assay. Protocol A for a manual luminometer was followed (protocol available at http://www.promega.com/tbs/tb281/tb281.html).

2.2.2.1.1 Lysis of Cells using Reporter Lysis Buffer

Four volumes of sterile mQ water was added to one volume of the 5x reporter lysis buffer. Lysis buffer was equilibrated to room temperature before use. The growth medium was removed from the reporter gene transfected 'mTS-pTetOn' cells (section

2.2.1.7.6) and cells rinsed in PBS being careful not to dislodge cells. As much PBS as possible was then removed by aspirating the PBS, leaving the plate tilted to drain and aspirating again. 200µl of 1x reporter lysis buffer was added to each of the wells and the cells were left slowly rocking on a plate rocker (Ratek Instruments, Australia) for 15 minutes at room temperature. The wells were then scraped using a sterile 1ml pipette tip and the cell lysates transferred to a microcentrifuge tube on ice. Cell lysates vortexed and then frozen at -80°C until the day of the assay (within 2 weeks). On the day of the assay the lysates were thawed at room temperature and vortexed for 15 seconds. They were then centrifuged at 12,000xg for 2 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube and kept on ice.

2.2.2.1.2 Preparation for the assay

The lyophilized luciferase substrate was reconstituted in the luciferase assay buffer. Unused reconstituted substrate was aliquoted and stored at -80°C. Reconstituted substrate was equilibrated to room temperature and mixed well before use. It is also light sensitive and therefore protected from light as much as possible.

2.2.2.1.3 Manual Luminometer protocol

A Turner Design manual luminometer (Alphatech systems ltd, Auckland) was used to measure the bioluminescence of the samples. The assay was carried out at room temperature, so both Luciferase substrate and cell lysates were equilibrated to room temperature before beginning. The luminometer had previously been calibrated so that an error reading would show at signal saturation intensities. Before beginning the assay the inducible positive control was tested, this will give the highest reading possible. If this sample was still within in the linear range duplicates of 10µl of cell lysate for each sample were pippetted into clear 1.5ml microcentrifuge tubes. If it wasn't cell lysates were diluted 1 in 10 in reporter lysis buffer and then 10µl duplicates dispensed. 50µl of the luciferase assay reagent was added to the sample. The mixture was pipetted 2-3 times to mix then immediately placed in the luminometer. The luminometer was programmed to have a 2 second delay followed by a 10second measurement of activity. Light intensity production should be constant for about 1 minute after substrate addition before decaying away. The measurement for each sample was recorded and the average taken for use in normalizing calculations with β-Galactosidase enzyme assay measurements.

2.2.2.2 β-Galactosidase Enzyme Assay System

All components for this assay and the protocol followed were from the β-Galactosidase Enzyme Assay Kit using protocol B (Promega, USA, protocol available at www.promega.com/tbs/tb097/tb097.pdf). The β-Galactosidase enzyme hydrolyses colourless ONPG (*o*-nitrophenyl-β-D-galactopyranoside) to *o*-nitrophenol which is yellow and is measured using spectrometry. This assay was done on the same day as the luciferase assay and measurements taken were used to normalise results from the luciferase assay for cell number and transfection efficiency.

2.2.2.2.1 Lysis of Cells with Reporter Lysis buffer

See section 2.2.2.1.1.

2.2.2.2.2 β-Galactosidase 96-well plate assay

The 2x assay buffer was thawed and then stored on ice. 50µl, 30µl and 10µl of one of the sample lysates was put in 3 wells of a 96well plate on ice and the total made up to 50µl with reporter lysate buffer. 50µl of 2x assay buffer was added and then the plate covered in parafilm and placed in a 37°C incubator (Sanyo) for 30 minutes. The plate was checked to see development of a faint yellow colour and the optimum concentration of lysate was determined. Duplicates of cell lysate samples were put into the wells and made up to 50µl using reporter lysis buffer. 50µl of 2x assay buffer was added and the plate incubated for 30min at 37°C. The plate was then read using a Versa max microplate reader (Molecular Devices, USA) and accompanying software (Softmax® Pro, Molecular Devices, USA). The measurement was carried out at a wavelength of 405nm. Average values for each sample were used to normalize luciferase results.

2.2.2.3 Dual-Luciferase Reporter Assay System.

The components of this system were all supplied in the dual luciferase reporter assay system kit (Promega, USA) The standard protocol for a manual luminometer was followed (available at www.promega.com/tbs/tm040/tm040.pdf). In this assay firefly luciferase expression is normalized to *Renilla* luciferase expression and each luciferase is measured sequentially from a single sample. The system works because although both enzymes produce photons of light measured in a luminometer they use different substrates; firefly luciferase oxidizes beetle luciferin, while Renilla luciferase oxidizes

coelenterate-luciferin. All components were stored at -20°C except reconstituted luciferase assay substrate which was aliquoted and stored at -80°C.

2.2.2.3.1 Lysis of Cells using Passive Lysis Buffer

Four volumes of sterile mQ water was added to one volume of 5x passive lysis buffer. Passive lysis buffer was equilibrated to room temperature before use. The growth medium was removed from the reporter gene transfected 'mTS-pTetOn' cells (section 2.2.1.7.6) and cells rinsed in PBS being careful not to dislodge cells. As much PBS as possible was then removed by aspirating the PBS, leaving the plate tilted to drain and aspirating again. 200µl of 1x passive lysis buffer was added to each of the wells and the cells were left slowly rocking on a plate rocker (Ratek Instruments, Australia) for 15 minutes at room temperature. Cell lysates were transferred to microcentrifuge tubes on ice and vortexed they were then centrifuged at 4°C at 12,000xg (epindorf centrifuge 5415D, Germany) for 30 seconds and lysates transferred to a new tube. Cell lysates were frozen at -80°C until the day of the assay (within 2 weeks). On the day of the assay the lysates were thawed at room temperature and vortexed.

2.2.2.3.2 Preparation for the assay

The lyophilized luciferase substrate was reconstituted in the luciferase assay buffer II. Unused reconstituted substrate was aliquoted and stored at -80°C. Reconstituted substrate was equilibrated to room temperature and mixed well before use. It is also light sensitive and therefore protected from light as much as possible. Before each assay fresh Stop & Glo reagent was prepared. 1 volume of 50x Stop & Glo Substrate was added to 50 volumes of Stop & Glo Buffer in a siliconised microcentrifuge tube to give enough for 50µl of reagent per assay, this was also equilibrated to room temperature.

2.2.2.3.3 Dual Luciferase Assay in a Manual Luminometer

50µl of luciferase assay reagent II was predispensed into 2 clear microcentrifuge tubes for each cell lysate sample. 10µl of room temperature cell lysate was added to the tube, pipetted to mix and then immediately placed in a TD-20 luminometer (Alphatech systems ltd, Auckland). After a 2 second delay a 10 second period measurement was taken. The firefly luciferase measurement was recorded and then the tube removed and 50µl of Stop & Glo reagent added. The Stop & Glo reagent quenches the firefly reaction and activates the *Renilla* luciferase enzyme within 1 second. The tube was replaced and after a 2 second delay a further 10 second measurement was taken. If an

error message on the luminometer was given due to saturation, the cell lysate was diluted 1 in 10 in 1x passive lysis buffer and the readings repeated. The *Renilla* measurement was then used to normalize the firefly measurement.

All reagents were thoroughly mixed by vortexing (except enzymes) and then briefly centrifuged before use. Enzymes were briefly centrifuged before use and kept in a -20°C cold block for a minimum amount of time whilst they were in use.

2.2.2.4 Transformation of Bacteria with plasmid DNA

In order to propagate a particular plasmid for use in later down stream applications an intact plasmid was transformed into commercially available competent *E.Coli* strain DH5α cells with an efficiency of $1x10^6$ transformants/μg of DNA (Invitrogen Corporation). Two 50μl aliquot of DH5α cells were removed from -80°C storage and thawed on ice. 1μl of 1ng/μl plasmid DNA was added to one of the aliquots the and gently tapped to mix. This was left to incubate on ice for 30 minutes. The other aliquot was used as a negative control and did not receive any DNA. After the incubation both tubes were heatshocked at 42°C for 75seconds. Cells were then plunged into ice and allowed to cool for 2 minutes. 1ml of SOC medium (Invitrogen Corporation) was added and the cells put into a 37°C shaking incubator (300rpm) for 60 minutes (New Brunswick Scientific, USA). 100μl of the transformed cells were aseptically spread onto an LB-ampicillin plate. The tubes were centrifuged at 10,000rpm for 1 minute (Biofuge pico Heraeus, Germany) and 800μl of supernatant removed. The rest of the contents of each one were aseptically spread on LB-ampicillin plates. Plates were allowed to dry and then incubated upside down overnight (~16 hours) in a 37°C incubator (Sanyo). The following morning the negative control plate was checked to ensure no colonies had grown, and then an isolated colony of transformed bacteria was selected using a sterile 200μl pipette tip.

2.2.2.5 Maxipreparation of Plasmid DNA

The QIAGEN Plasmid Maxi Kit was used for preparation of high quantities of plasmid DNA (QIAGEN, USA). The manufacturer's protocol was followed with some modifications. All reagents were supplied in the kit unless otherwise stated.

2.2.2.5.1 Growth of Bacterial cells

A colony of bacteria containing the plasmid was picked using a sterile pipette tip and used to inoculate a starter culture of 3mls of LB + 100μg/ml ampicillin in a sterile 13ml snap-cap tube (Sarstedt, Germany) and grown for the day at 37°C in a shaking incubator (~7 hours). This was added to 250ml of LB + ampicillin in a 1 litre flask and grown overnight (~16 hours) at 37°C in a shaking incubator at 300rpm (Edison, New Jersey, USA).

2.2.2.5.2 Cell Harvesting and lysis

The cells were transferred to a 250ml centrifuge tube and centrifuged at 6000 x *g* for 15minutes at 4°C (Sorvall RC5C, USA). The supernatant was discarded and centrifuge tube left to drain upside down to remove all traces of supernatant. The pellet was resuspended in 10ml of buffer P1 which contained 100μg/ml of RNase. The pellet was thoroughly resuspended by pipetting up and down. 10ml of buffer P2 was added and gently mixed by inverting 4-6 times to avoid shearing of genomic DNA.

2.2.2.5.3 Purification of Plasmid DNA

Immediately after, 10ml of chilled (4°C) P3 buffer was added. This was also mixed gently by inverting 4-6 times and then the tube was left to incubate on ice for 20minutes. A precipitate of genomic DNA, proteins and SDS forms. The sample was mixed again and then centrifuged at 16,000 rpm using a SS-34 rotor (Sorvall RC5C, USA) for 30 minutes at 4°C. The clear supernatant was transferred to a new centrifuge tube and centrifuged again at 16,000 rpm for 15minutes at 4°C. During this time a QIAGEN-tip 500 column was equilibrated using 10ml of buffer QBT, flow through was discarded. The clear sample supernatant was promptly loaded on the column and allowed to flow through under gravity flow. The column was then washed twice with 2x 30ml lots of buffer QC and flow through discarded. Plasmid DNA was then eluted with 15ml of buffer QF. The flow through was collected into a 30ml centrifuge tube. DNA was precipitated by adding 10.5ml of isopropanol (BioLab Australia Ltd) and mixing. The solution was centrifuged at 11,000rpm in a SS-34 rotor for 30minutes at 4°C. The supernatant was carefully removed so as not to disturb the DNA pellet and the pellet was washed with 5ml of room-temperature 70% ethanol (BioLab Australia Ltd) and spun again at 11,000rpm for 15 minutes at 4°C. The ethanol was removed using a pipette and then the pellet left to air dry for 5-10minutes. The DNA pellet was resuspended in 100-300μl of TE buffer.

2.2.2.6 Agarose Gels

Agarose powder (Invitrogen life Technologies, NZ) was added to 0.5 x TAE buffer, heated to dissolve in a microwave and cooled before adding 10μg/ml ethidium bromide (Sigma-Aldrich, USA) to generate a final concentration of 0.5ng/ml. Gels were 1% 1.5%, 1.7% or 2% depending on the expected size of the DNA fragments analysed. The melted agarose was then poured into a block containing a comb and allowed to set. For RNA samples gel tanks and all blocks/combs to be used were first treated with 3% H_2O_2 (Sigma-Aldrich, USA) for 2 hours to remove RNases. Gels were run at 5-10 volts per cm until satisfactory separation of bands could be seen. Ethidium bromide staining was visualized using ultraviolet light and gel images were generated using the Quantity One Gel Doc system (Bio-Rad Laboratories, 2001, USA).

2.2.2.7 Restriction Enzyme Digest of Large Amounts of Plasmid DNA

To prepare large amounts of plasmid cut with a restriction enzyme (in general this was used to linearise plasmids for transfection) the following general protocol was followed: to 30μg of DNA (x μl) was added 20μl of the appropriate restriction buffer, H_2O to give final total of 200μl and 5μl of restriction enzyme (10U/μl). An Important consideration in performing restriction enzyme digests is that the concentration of the enzyme is not too high in the reaction mix. This is because the enzymes are stored in 50% glycerol and a final concentration of glycerol greater than 5% in the reaction mix can inhibit the digestion of the enzymes and also cause unspecific cleavage of DNA ("star" activity). The restriction enzyme was always added last to the reaction mix and mixed by pipetting up and down; avoiding production of air bubbles. The tube was placed in a thermomixer (epindorf thermomixer compact, Germany) at the appropriate temperature (generally 37°C) for 1-2hours. A phenol/chloroform extraction was done by adding 200μl phenol/chloroform (Invitrogen life technologies, NZ), vortexing and centrifuging (Biofuge pico Heraeus, Germany) at maximum speed for 5 minutes. The supernatant was transferred to a fresh tube and 20μl 3M sodium acetate (pH 5) and 300μl of ethanol added and vortexing. The tube was centrifuged (epindorf 5415D, Germany) at 4°C for 15 minutes at maximum speed, the supernatant was discarded and the pellet washed with 0.5ml 70% ethanol, centrifuged again at max speed, 4°C, and the ethanol aspirated. The DNA pellet was allowed to dry by leaving the cap off at

room temperature for 5 minutes, resuspended in 25µl T0.1E and then the concentration measured using the nanodrop.

2.2.2.8 Restriction Enzyme Digest of Small Amounts of DNA

About 1µg of DNA was cut in a 20µl digest mixture which included 2µl of the appropriate buffer (10x, supplied with the enzyme), 1µl of restriction enzyme (10U/µl) and sterile water to make the total volume up to 20µl. This was allowed to digest for 1hour at 37°C.

2.2.2.9 PCR Amplification of DNA

For a 25µl PCR reaction about 100ng of genomic DNA or 0.1ng of a plasmid was used for the initial quantity of template DNA. This was added to 2.5µl of 10x PCR buffer containing $MgCl_2$ (supplied with FastStart Taq DNA polymerase; Roche, Germany), 0.5µl each of a working solution of the forward and reverse primers, 0.5µl of 10mM dNTP's (Roche), PCR grade water (Roche, Germany) to 25µl, 0.2µl of FastStart Taq DNA polymerase (Roche, Germany). This was placed in a thermocycler (BioRad, Australia) with the following programme: 95°C for 7 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 40 seconds and 72°C for 1 minute. A final extension of 72°C for 7 minutes was then run.

2.2.3 mTS Analysis of Gene Expression Using Real-Time PCR

Analysis of mTS cell gene expression after media treatments (section 2.2.1.10) or siRNA mediated Elf5 knockdown (section 2.2.1.7.3) was performed by extracting RNA extraction from mTS cells using TRIZOL reagent (invitrogen life technologies), removing contaminating DNA, reverse transcribing purified mRNA to cDNA and then measurement of relative target gene expression by real-time PCR. During RNA extraction and handling special care was taken to avoid contamination of samples with RNases. This included wiping down surfaces and pipettes with RNase away (Molecular Bioproducts Inc., USA) wearing powder free gloves, using filter tips and ensuring all reagents and consumables were RNase free. DEPC treated water was used and all RNA samples stored at -80°C. The first section gives the original protocol used when work was first started. The second section gives the modified protocol. Rationale for modifications is given in Chapter 3.

2.2.3.1 mTS analysis of Gene Expression: Original Protocol

This protocol is an 'inhouse' protocol which was currently in use when I started work; therefore initial attempts at quantifying gene expression were carried out using this protocol.

2.2.3.1.1 Cell Harvesting for RNA Extraction

Cell growth media was aspirated and cells washed in PBS (Oxoid) by adding 2-3mls of PBS per $10cm^2$ well, rotating the dish and then aspirating PBS. 0.6mls of TrypLE (Gibco) was added to each well the plate returned to the 37°C incubator for 10 minutes. 0.8mls of mTS media was added to neutralize the TrypLE enzyme and the contents of each well was pipetted up and down to ensure all cells were dissociated from the bottom. The cell suspension was then transferred to a microcentrifuge tube and centrifuged (epindorf centrifuge 541D, Germany) at 4°C for 5 minutes at 4000 rpm. The supernatant was discarded and the cells resuspended in 400μl of TRIZOL (Invitrogen Life Technologies, NZ). The cell pellet was vortexed and pipetted up and down through a 200μl pipette tip to thoroughly dissolve the cells.

2.2.3.1.2 RNA extraction

80μl of Chloroform (MERCK, Darmstadl, Germany) and 1μl of RNase free 10mg/ml glycogen (Roche Diagnostics, Germany) was added to each sample. Glycogen acts as a carrier to the aqeous phase and is co-precipitataed with RNA (Invitrogen life technologies, 2003). Samples were vortexed for 30 seconds and then centrifuged (epindorf centrifuge 541D, Germany) for 5 minutes at maximum speed to separate the phases. The upper aqueous layer was transferred to a new tube and then 200μl of ice-cold isopropanol (BioLab Australia Ltd) added. Samples were vortexed to mix and then placed in a -20°C freezer and RNA precipitated overnight. The RNA was pelleted by centrifugation at max speed in a microcentrifuge at 4°C for 15minutes. The supernatant was aspirated and a small while glycogen pellet could be seen. The pellet was washed in 100μl of 75% ethanol (BioLab Australia Ltd) and spun again at maximum speed for 10 minutes at 4°C. The ethanol was aspirated and then the pellet allowed to air dry for 5 minutes. The RNA was resolubilised in 15μl of DEPC treated H_2O.

2.2.3.1.3 DNAase treatment

This step is necessary for real-time PCR when amplicons do not span introns and to avoid the amplification of potential pseudogenes in contaminating genomic DNA. A master mix per tube of 1µl DTT (Invitrogen Life Technologies, NZ), 2µl of 10x DNAseI reaction buffer, 1µl of RNaseOut (Invitrogen Life Technologies, NZ) and 1µl of amplification grade DNase I (Invitrogen Life Technologies, NZ) was added to each sample. The samples were placed at 37°C for 30minutes (epindorf thermomixer compact, Germany). After DNase and an equal volume (20µl) of phenol/chloroform (Invitrogen life technologys,NZ) and 2µl of 3M sodium acetate (pH 5) was added. Samples were vortexed and centrifuged (epindorf centrifuge 541D, Germany) for 10min at maximum speed at 4°C for 5 minutes and the top layer transferred to a new tube. 120µl of ethanol (BioLab Australia Ltd) was added and the RNA precipitated at -20°C for 2-3hours. The RNA was pelleted by centrifugation (epindorf centrifuge 541D, Germany) at maximum speed for 15minutes at 4°C and then washed with 200µl of 75% ethanol and the centrifugation step repeated. The 75% ethanol was aspirated and the pellet dried for 5 minutes before resuspending it in 11µl of DEPC treated H_2O. RNA was stored at -80°C.

2.2.3.1.4 Reverse Transcription

10µl of each RNA sample was transferred to a thin-walled PCR tube and to this was added 1µl of Oligo(dT)12-18 primer (500ng/µl; Invitrogen Life Technologies, NZ) and 1µl of 10mM dNTP mix (10mM each nucleotide; Invitrogen Life Technologies, NZ). This was put into a thermocyler (PTC-200, MJ Research, USA) with heated lid and set to 65°C for 5 minutes to denature the RNA. The tubes were immediately transferred to ice and rapidly chilled for at least two minutes. A master mix was prepared on ice consisting of 4µl 5x 1st strand buffer (supplied with Superscript III, Invitrogen) 1µl 0.1 M DTT (supplied with Superscript III)1 µl RnaseOut (Invitrogen life technologies, NZ) and 1 µl Superscript III (Invitrogen life technologies, NZ) and added to each tube. The PCR tubes were spun (Labnet International Inc, USA) to collect their contents to the bottom and then returned to the thermocycler. They were then incubated for 1 hour at 50°C followed by 15 minutes at 70°C to stop the reaction; the cDNA produced was stored at -20°C.

2.2.3.2 mTS analysis of Gene Expression: Modified Protocol

2.2.3.2.1 Cell Harvesting for RNA Extraction

This protocol is based on the manufacturer's protocol for TRIZOL (Invitrogen Life Technologies, , 2003) for a 10cm^2 well: Cells were rinsed with PBS; this is aspirated, the plate is left tilted to drain further and any residual PBS is aspirated. 1ml of TRIZOL was then added to each well. The TRIZOL was then vigorously pippetted up and down using a 1ml pipette tip to aid in dissolving the cells. It is important this step is carried out as rapidly and thoroughly as possible to avoid degradation of mRNA which accounts for only 1-5% of a cells total RNA. TRIZOL contains phenol and guanidinium isthiocyanate. Guanidinium isthiocyanate disrupts cells, solubilises their components and denatures endogenous RNAses (Sambrook & Russel, 2001b). Cell homogenates were stored at -80°C until RNA isolation could be performed.

2.2.3.2.2 RNA extraction

0.2ml of Chloroform (MERCK, Darmstadl, Germany) and 1µl of RNase free 10mg/ml glycogen (Roche Diagnostics, Germany) was added to each sample. Glycogen acts as a carrier and has been shown to improve RNA yields. The samples were vortexed for 15 seconds. They were then centrifuged at 12,000 x g for 15 minutes at 4˚C. The RNA goes into the aqueous layer on the top, while proteins accumulate at the aqeous/organic interface. The top layer (~600µl) was transferred to a fresh microcentrifuge tube. 0.5ml of isopropanol alcohol was added (BioLab Australia Ltd) and then the samples vortexed to mix. The samples were then put in a -20˚C freezer overnight to allow for precipitation of the RNA. The samples were centrifuged at 12,000 x g for 15 minutes at 4˚C and the supernatant aspirated using an RNase free 200ul pipette tip being careful to avoid the pellet. The pellet was washed with 1ml 70% ethanol (BioLab Australia Ltd) and then centrifuged again at 4˚C for 5 minutes at 7500 x g. The alcohol was aspirated carefully and the samples briefly centrifuged to collect any remaining alcohol. Any visible droplets of alcohol were removed as alcohol can inhibit down stream enzymatic reactions. The samples were left at room temperature with the caps off to dry and then resuspended in 28µl of DEPC treated H2O.

2.2.3.2.3 DNase treatment

As in section 2.2.3.1.3 except the volumes for the components DTT, DNase reaction buffer and RNaseOut were doubled and the volume of DNase I was increased to 4µl.

These were added to the 28µl RNA sample to give a total volume of 40µl. The time for the digestion was increased to 1 hour at 37°C. The volumes of Phenol/chloroform and sodium acetate were also doubled. The concentration and quality of the RNA was routinely checked using the NanoDrop spectrometer (Nanodrop technologies, USA).

2.2.3.2.4 Reverse Transcription

As in section 2.2.3.1.4, however the volume of RNA sample was reduced to 1µl and 11µl of DEPC water was added at the first step before denaturation at 65°C.

2.2.3.3 SYBER-Green Real-time PCR

Expression of target genes was assessed by relative quantitative real-time PCR. (qRT-PCR). A target gene was amplified in "real time" and the number of cycles taken for the fluorescence of SYBER-Green to reach a certain threshold (Ct) recorded, using an ABI 7900 HT (Applied Biosystems, Singapore) or later the 'takeoff' cycle was recorded when using the Rotor-Gene 6000 (Corbett Life Science, Australia). The gene target Ct or take off was then normalized to a reference gene; a gene whose expression is thought to remain stable in the different samples compared. Several different algorithms were used to normalize gene expression and are discussed in chapters 3. In preparing qRT-PCR runs all reagents were thoroughly mixed and briefly centrifuged in a minicentrifuge (BioRad, USA) before use. Master mixes and samples were kept on ice and kept away from light to avoid SYBER-Green dye bleaching. qRT-PCR reaction mixtures and set up was carried out just prior to each run to avoid primer-dimers. Filter tips were also used throughout to avoid contamination as this is a very sensitive technique.

2.2.3.3.1 Reverse Transcription Negative Control

1ul of the DNase treated RNA for each sample was diluted in 19µl of TE buffer to make a reverse-transcription negative control (RT-). This control was used run in at least one real-time PCR assay to check for contamination of the RNA sample with genomic DNA (i.e. an incomplete DNase reaction). Preferably a gene primer set was chosen which did not cover introns in screening the RT- controls.

2.2.3.3.2 Sample preparation

Sample cDNA was diluted 1/20 and 1/40 in PCR grade water (Roche, Germany). One sample also had dilutions made up of 1/80 and 1/160 and was used to determine the

amplification efficiency (not required when using the Corbett). Diluted cDNA was stored at 4°C short term (less than a week) when samples were being continuously used to avoid multiple freeze/ thaw cycles. Longer term diluted cDNA storage was at -20°C.

2.2.3.3.3 qRT-PCR Primer Design and Testing

Publicly available mouse sequence data was used to generate primers for amplification of a selected gene (available from http://www.ensembl.org/Mus_musculus/index.html.) A gene name was searched in http://www.ncbi.nlm.nih.gov/sites/entrez under nucleotide or in the case of the microarray the Affymetrix probe number was matched to a gene using Ingenuity Pathway Analysis 4.0 version 3. When available the mRNA reference sequence was used (NM_#######). The mRNA sequence was pasted into BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start) and a search performed against the mouse genome to find the location of introns. The mRNA sequence was then used to design primers which where possible the amplicon spanned an intron to ensure any contaminating genomic DNA was not amplified. Primers were also designed to amplify the 3' end of a gene so as to minimize the impact of reverse transcription inefficiencies. For use in the ABI 7900 qRT-PCR machine primers were kindly designed by Peter Pfeffer using GCG (eXcursion software, version 5.1B, 2003, Hewlett-Packard Development Company, California, USA). The criteria used for primer design was a length between 18-22 bases, 40-50% GC content, melting temperature between 50°C-65°C together with a PCR product length between 200-500bp. Primers designed for the Corbett machine used the Vector NTI 10.1.1 (Invitrogen Corporation, 2005) software, the same criteria was applied except that Corbett PCR product length was designed to be 200-300bp in due to the shorter time allowed for extension. The 3' end of the primers was chosen so the last 5 base pairs contained a maximum of two G's or C's (the GC clamp). Primers were prepared as given in section 2.1.3.6. Before using a new primer pair for analysis each primer set was run in trial qRT-PCR reaction using one of the cDNA samples. The melting curve was checked to ensure only one product was formed; the DNA product of the reaction was run on an agarose gel and checked for correct size, and a single band.

2.2.3.3.4 qRT-PCR using the ABI 7900 HT Machine

Each run was carried out in a MicroAmp Optical 96-well plate (Applied Biosystems, USA). A master mix was prepared for each primer pair and the reference gene which consisted of: 7.5µl of SYBER-Green master mix (Applied Biosystems, USA), 6.2µl of PCR grade water (Roche, Germany), 0.1µl forward primer, 0.1µl reverse primer, 0.1µl Taq Fast start (Roche Diagnostics, Germany). This was pipetted to mix and then 14µl pipetted into each well. Triplicates were done for each sample/gene combination; two using a cDNA concentration of 1/20 and one 1/40. A dilution series was also included for each gene and used to calculate the amplification efficiency (used in the normalisation calculations). Each run also included a negative control of PCR grade water instead of template cDNA to check for contamination. An optical adhesive cover (Applied Biosystems, USA) was applied to the plate and then the plate was spun in a salad mixer. The plate was put into the ABI machine and the program run: 50°C for 2 minutes (UTP degrading enzyme works), 95°C for 5 minutes (activation of fast start TAQ polymerase) followed by 40 cycles of 95°C for 15 seconds (denaturation), 56°C for 30 seconds (annealing), 72°C for 30 seconds (DNA polymerization) and 78°C for 10seconds (SYBER-Green fluorescence measurement taken). Then a dissociation analysis was done: 95°C for 15 seconds followed by 60°C for 15 seconds (to allow the PCR product to anneal) then slowly ramping up to 95°C measuring fluorescence at every °C. After the run the data was analysed using ABI software, with an automatic threshold of 0.01 used. Data was exported to an Excel spreadsheet (Microsoft Office 2003)for analysis.

2.2.3.3.5 qRT-PCR using the Rotor-Gene 6000

Triplicates for each sample/gene combination of two times 2µl of 1/20 diluted cDNA and one 1µl diluted cDNA + 1µl of PCR grade water (Roche, Germany) were pipetted into each tube of either a 72 well rotor or a gene-disc 100 well rotor (Corbett life Science, Australia). Each run also included a negative control of PCR grade water instead of template cDNA for each primer pair used. A master mix was prepared of 5µl lightcycler 480 SYBER Green I Master (Roche, Germany), 0.1µl forward primer, 0.1µl reverse primer and 2.8µl of PCR grade water. This was mixed and then 8µl aliquoted into each tube. The program run was: 10 minutes at 95°C then 40 cycles of: 10 seconds at 95°C, 15 seconds at 60°C (or other depending on primers), 20 seconds at 72°C followed by a dissociation curve of 72°C for 90 seconds then 72°C to 95°C at 1°C every 5 seconds. Rotor-gene 6000 series software (version 1.7, Corbett research,

Australia) was used to analyse the raw data. The Takeoff point was exported to Excel (Microsoft Office, 2003) for each reaction. "The Takeoff point is defined as the cycle at which the second derivative (of the amplification plot) is at 20% of the maximum level, and indicates the end of the noise and the transition into the exponential phase." (Rotor –gene 6000 series software help)

This was treated the same way as a Ct value.

2.2.3.3.6 qRT-PCR Analysis

After each run several parameters were checked to ensure the validity of the run:

i. That the Ct (takeoff) for the water control was near 40 to indicate there was no contamination.
ii. That the RT- control for each sample is at least 5 cycles higher than samples using a primer set for an intron-free amplicon (e.g. actin primers). If there was a similar Ct to samples, then the samples were discarded.
iii. The melt curve was checked to ensure a single peak was observed (single product)
iv. Duplicate variation should vary by less than 0.2 Ct units
v. The 1:2 dilution of a sample should have a Ct (takeoff) between 0.6-1.4 units greater than the 1:1 that is higher than Ct of undiluted sample as determined by reaction efficiency. If the difference was outside this boundary it indicates that one is no longer measuring in the linear range of the PCR curve and the sample was discarded from analysis.

Relative PCR compares the difference between a treated sample and a control sample for a particular gene. The relative expression of each gene was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), except that a gene specific amplification efficiency was used instead of 2. Each gene is first normalized by dividing its expression by a reference or housekeeper gene which should not be affected by any of the treatments studied. The efficiency of the reaction (*a*) was found by either using as dilution series of one of the samples and graphing Log[sample/plasmid/amplicon concentration] versus Ct; then efficiency can be calculated by :

$$a = 10^{-(slope\ of\ (log[conc])/Ct).} \quad \text{Equation 1}$$

On the Rotor-gene 6000 machine the efficiency is calculated for each individual reaction tube using the accompanying software (Corbett, Australia) and the average efficiency for all the reaction tubes of a particular primer set was used in copy number the calculations. The efficiency should be greater than 1.6 but not greater than 2.1

The amplification efficiency was then used to calculate a 'copy number' (N_I) for each sample for the gene of Interest, and the reference gene (N_R). Copy number is given by the equation:

$$N=a^{-Ct} \qquad \text{Equation 2}$$

Each gene of interest copy number (N_I) is then normalised against the reference gene copy number (N_R) or the geometric mean (N_g) of many reference genes may be used:

$$N_g = \sqrt[n]{N_1 N_2 N_3 ... N_n} \qquad \text{Equation 3}$$

Where n is the number of housekeepers used and N_n is the copy number of the nth reference gene.

The normalised gene copy number (X_I) is given by:

$$X_I = \frac{N_I}{N_R} \qquad \text{Equation 4}$$

X_I is then divided by the normalised gene copy number of the control sample (X_{IC}) to give the relative expression for the gene of interest in the treated sample (R_I).

$$R_I = \frac{X_I}{X_{IC}} \qquad \text{Equation 5}$$

2.2.4 Subcloning

Subcloning is the transfer of a gene of interest from one vector construct to another. The exact protocol depends on the restriction enzyme sites available and their compatibility in the vector containing the gene of interest and then vector to which the gene will be ligated into.

2.2.4.1 Subcloning strategy

The vector constructs containing the DNA fragment and the target vector were analysed for restriction sites which occur in the same 5' to 3' order to see if compatible sites were available. If the ends of the target vector and the DNA fragment are not compatible then 'sticky' ends may be 'blunted' by filling in the 3' recessed ends using klenow enzyme (Roche, Germany).

2.2.4.2 Preparation of DNA Fragment of Interest for Ligation

2.2.4.2.1 Restriction Enzyme Digest

The original vector was digested using the restriction enzymes decided on according to the protocol given in section 2.2.2.8.

2.2.4.2.2 Klenow 'Fill in' of Sticky Ends

To fill in the sticky ends (if required) the Klenow enzyme (Roche, Germany) was used. This enzyme is the large fragment of DNA polymerase I after digestion with subtilisin protease. It catalyses the addition of deoxynucleoside-5'-triphosphates (dNTPs) to the 3'-hydroxyl terminus of a primer-template DNA and also retains 3' to 5' exonuclease activity, however it lacks 5' to 3' exonuclease activity (information and protocol available from Roche: www.roche-applied-science.com/pack-insert/1008404a.pdf). It therefore fills in recessed 3' ends or digests away protruding 3' ends to give blunt ends. To the restriction enzyme digest mixture (20μl) was added 2.3μl of a mixture of all four dNTPs each at 10mM and 1μl of Klenow enzyme. This was incubated at 37°C for 30 minutes, then the enzyme heat killed by heating to 65°C for 10 minutes.

2.2.4.2.3 Geneclean DNA purification from Solutions

To prevent the Klenow enzyme filling in desired sticky ends, compatible restriction enzyme digests were performed after blunting of incompatible ends and after the Klenow enzyme had been removed using the Geneclan kit (QBiogene, USA). The

protocol from the kit was followed (Geneclean II kit, QBiogene, USA); all reagents were supplied in the kit unless specified, the kit was stored at 4°C. The glass milk was first vortexed vigorously for 1minute to suspend the silica particles. Three volumes of NaI added were added to the DNA mixture, then 5μl of glass milk was added and the mixture vortexed gently. This was left to incubate at room temperature for 5 minutes to allow DNA to bind to the silica particles, mixing again after 2 minutes to keep the silica particles in suspension. The glassmilk bound to the DNA was then pelleted by centrifuging for 5 seconds at full speed (Biofuge pico Heraeus, Germany). The supernatant was aspirated and the pellet resuspended in 500μl of icecold NEW Wash by gently pipetting up and down with a 1ml pipette. The NEW Wash solution had previously been made up with ethanol (BioLab Australia Ltd) according to the manufacturer's instructions. This was centrifuged for 5 seconds at maximum speed, the supernatant discarded and then the wash and centrifuge repeated. After the second wash the tube was again briefly centrifuged and all traces of ethanol removed. The pellet was dried for 5 minutes at room temperature with the cap removed then 5μl of DEPC water was added to elute the DNA. This was mixed gently by pipetting up and down. Finally the tube was centrifuged for 30 seconds at maximum speed and the supernatant containing the eluted DNA transferred to a new tube. After this the second restriction enzyme digest was performed.

2.2.4.2.4 Separation of Target Fragment

The DNA fragment of interest was then separated from the vector by running the restriction enzyme digest on an agarose gel containing ethidium bromide (section 2.2.2.6). The desired band was cut out of the gel by viewing the gel under low powered UV light (to avoid strand breaks). A scalpel blade cleaned with ethanol was used to rapidly cut the band out, minimizing UV light exposure. The gel slices were placed in pre-weighed microcentrifuge tubes. The DNA was then purified from the gel using the Geneclean kit (QBiogene, USA).

2.2.4.2.5 Geneclean DNA Purification from Agarose Gels

The protocol from the kit was followed (Geneclean II kit, QBiogene, USA); all reagents were supplied in the kit unless specified, the kit was stored at 4°C. The gel slice containing the target DNA was weighed then 3 volumes of NaI added. This was incubated at 55°C for 5 minutes to dissolve the gel. If after 5 minutes the gel was not fully dissolved it was incubated for 1 minute longer. During melting of the agarose gel

the glass milk was vortexed for a minute. The gel mixture was mixed by tapping the tube and then 5µl of glass milk was added and the mixture vortexed gently. This was left to incubate at room temperature for 5 minutes to allow DNA to bind to the silica particles, mixing again after 2 minutes to keep the silica particles in suspension. The glassmilk bound to the DNA was then pelleted by centrifuging for 5 seconds at full speed (Biofuge pico Heraeus, Germany). The supernatant was aspirated and the pellet resuspended in 500µl of icecold NEW Wash by gently pipetting up and down with a 1ml pipette6. The NEW Wash solution had previously been made up with ethanol (BioLab Australia Ltd) according to the manufacturer's instructions. This was centrifuged for 5 seconds at maximum speed, the supernatant discarded and then the wash and centrifuge repeated. After the second wash the tube was again briefly centrifuged and all traces of ethanol removed. The pellet was dried for 5 minutes at room temperature with the cap removed then 5µl of DEPC water was added to elute the DNA. This was mixed gently by pipetting up and down. Finally the tube was centrifuged for 30 seconds at maximum speed and the supernatant containing the eluted DNA transferred to a new tube. The DNA was quantified using the Nanodrop (Nanodrop technology, USA)

2.2.4.3 Preparation of Target Vector for Ligation

The target vector had to be prepared to receive the DNA fragment, by first cutting with the appropriate restriction enzymes and then removing the 5' terminal DNA phosphosphate to prevent the vector joining to itself during the ligation step. Finally the cut vector was purified using the Geneclan kit (QBiogene, USA)

2.2.4.3.1 Restriction Enzyme Digest of Target Vector

The target vector was digested using the appropriate enzymes, for double digests the total volume of the reaction was increased to 30µl and a buffer which allowed both enzymes to work was used. If this was impossible then two sequential digests were performed, with the enzyme requiring the lowest salt concentration first.

2.2.4.3.2 Dephosphorylation of Cut Vector

To the restriction digest mixture was added mQ sterile water to give a total volume of 44µl, 5µl of 10x CIP buffer (supplied with CIP enzyme) and 1µl of 1U/µl Calf Intestine alkaline Phosphatase (CIP; Roche, USA). The reaction mix was incubated at 37° for 15minutes, followed by 15 minutes at 56°C, then 1µl of CIP was added and the

incubations repeated. The CIP was then heat inactivated by heating to 75°C for 10 minutes.

2.2.4.3.3 Purification of Cut Vector

The dephosphorylated cut vector was then run on an agarose gel containing ethidium bromide. A narrow band of the correct size cut out from the gel under UV light using a sterile scalpel blade and put into a pre-weighed microcentrifuge tube. The target vector was then purified from the gel using the Geneclean kit as described in section 2.2.4.2.5.

2.2.4.4 Ligation of DNA Fragment into Target Vector

This protocol is based on the manufacturer's protocol for T4 DNA Ligase (Invitrogen). A 3:1 molar ratio of insert to vector is suggested. To this was added 3μl of 5x Ligase reaction buffer (supplied with T4 Ligase; Invitrogen) and 1μl of T4 DNA Ligase (Invitrogen) and sterile mQ water to give a total volume of 15μl. This was incubated at 4°C for 24 hours (not 14°C as suggested in the protocol for a blunt ended ligation). A control ligation was also performed which was identical except that only vector DNA was used. This checks for the frequency of vector self ligation, i.e. how efficient was the dephosphorylation reaction by CIP.

2.2.4.5 Transformation

5μl of the ligation mix was added (in place of the 1μl of 1ng/μl DNA) to competent DH5α cells on ice and the transformation procedure given in section 2.2.2.4 followed.

2.2.4.6 Minipreparation of Ligated Vector

In the late afternoon (to prevent overgrowth of liquid cultures) 18 colonies were picked using sterile 200μl pipette tips and used to inoculate separate numbered 13ml snap cap tubes (Sarstedt, USA) containing 3ml LB/Amp broth. These were grown overnight at 37°C in a shaking incubator at 300rpm.The following day 1.5ml of culture was poured into labeled 1.5ml microcentrifuge tubes and then centrifuged for 1 minute at maximum speed (Biofuge pico Heraeus, Germany). The supernatant was aspirated (it is important to remove all supernatant as cell wall components present in the supernatant can inhibit the action of restriction enzymes) and then a miniprep was performed following the protocol given in "Molecular Cloning (Sambrook & Russel,

2001b). The solutions used were made according to the protocol given in molecular cloning (section 2.1.3.2.1) and the procedure was carried out with the tubes in ice: 100 µl of ice cold Solution I was added to each miniprep and vortexed vigorously until fully dissolved. 200 µl of freshly made Solution II was then added and mixed by inverting the tube 5 times. 150µl of ice cold solution III was added and the tubes inverted a few more times. The bacterial lysate was then centrifuged at maximum speed at 4°C (epindorf centrifuge 541D, Germany) for 5 minutes. The supernatant was transferred to a new tube and 450 µl of room temperature isopropanol (BioLab Australia Ltd) added. This was vortexed briefly before centrifuging again for 5 minutes at 4°C. The supernatant was aspirated taking care not to suck away the DNA pellet then the DNA was washed in 700 µl cold 70 % ethanol (BioLab Australia Ltd), centrifuged at maximum speed for 2 minutes and the ethanol aspirated trying to remove any traces of ethanol. The tubes were air dried by leaving them at room temperature for 3 minutes with the lids off and then the DNA resuspended in 30 µl TE containing 1:200 dilution of 10 mg/ml DF-RNAaseA (Invitrogen). This was incubated at 37°C for 30 minutes. The DNA can then be analysed by restriction digest using 3µl of sample in a 20µl reaction (section 2.2.2.8) and running on a gel to check for correct insert and orientation.

2.2.4.7 Preparation of a Glycerol Stock

One or two colonies analysed showing the correct restriction digest result were then made into a glycerol stock using some of the remaining bacterial culture: 150µl of pure sterile glycerol (Invitrogen) was added to 850µl of bacterial suspension in a cryovial. This was mixed and flash frozen by submerging in liquid Nitrogen (-196°) and then stored at -80°C, recording details as this is an ERMA requirement.

2.2.4.8 Sequencing

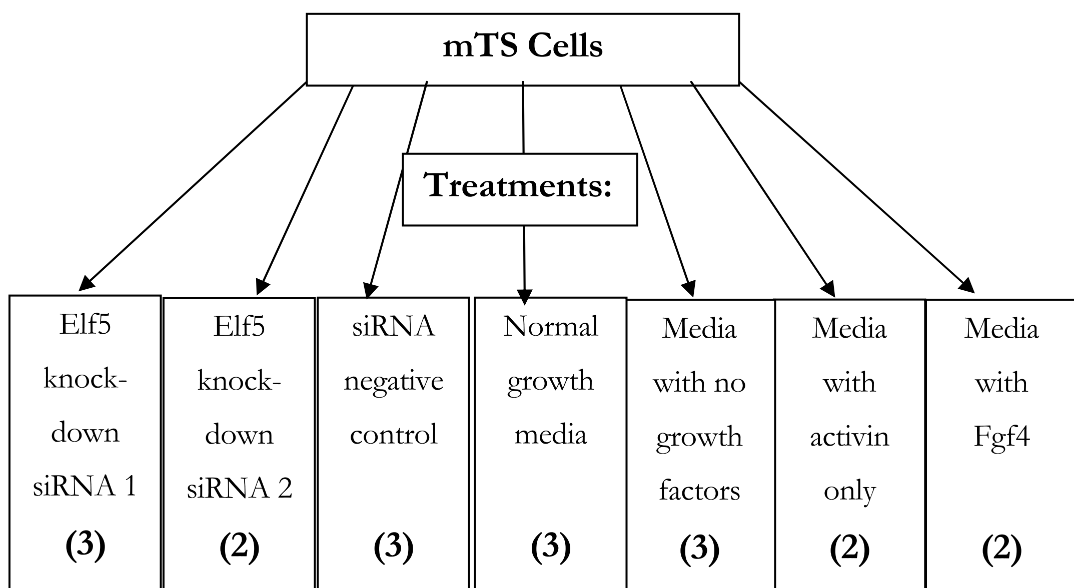
Selected colonies were used to inncoulate 250ml of LB/ampicillin broth and grown overnight to make a maxiprep. The DNA was then sequenced using two primers located before and after the insert. Sequencing was carried out by Waikato University DNA sequencing facility.

2.2.5 Microarray

The Affymetrix GeneChip® Expression platform was chosen for global analysis in the change of gene expression due to the various treatments detailed above. The array chosen was the GeneChip® Mouse Genome 430 2.0 Array. This array screens the expression of the entire mouse genome, and has 45,000 different probes. Cells were treated as specified in sections 2.2.1.7.3 and 2.2.1.10 with siRNA or by changing the media cells were grown in. RNA was then isolated from the cells following the modified RNA isolation protocol given in section 2.2.3.2. Following the DNAse treatment the 'One-Cycle Target Assay' protocol was followed closely adhering to the manufacturer's instructions. All reagents were supplied in the Affymetrix kits purchased unless specified.

2.2.5.1 Microarray Experimental Design

Three mRNA samples were pooled together for each microarray. And each treatment had either two or three microarrays performed ion each. Because Affymetrix arrays give an absolute expression value for each gene, any two arrays can be compared and all samples can be treated identically without the need for labeling samples to be compared with different dyes. A diagram of the different treatments and the number of arrays used for each treatment is given in brackets below:



2.2.5.2 Collection and Pre-Analysis of mRNA Samples

Cells were grown in six well plates and transfected or treated as given in the section on cell culture protocols. Because each treatment required between six and nine different samples (each well can be considered a separate experiment/sample) two six well plates of cells were treated. Isolation of mRNA from mTS cells was carried out according to the protocol given in section 2.2.3.2. 1µl of the sample was reverse transcribed as described in the protocol and used in real-time PCR analysis (section 2.2.3.3) using the ABI machine. The remainder of the RNA (10µl) was stored at -80°C.

1µl of RNA sample was removed form each stored sample and analysed on the Nanodrop (Nanodrop technologies). Before applying RNA samples to the Nanodrop the surface was wiped with RNase Away (Molecular BioProducts). RNA concentration and RNA quality were measured. The absorbance ratio A_{260}/A_{280} should be approximately 2.0 for pure RNA; values between 1.9 and 2.1 are acceptable (Affymetrix Inc., 2006a). The 1µl of RNA sample was then recovered from the Nanodrop and run on a gel, to check 18s and 28s rRNA bands could be clearly seen. An excess of samples meant any samples with low RNA quality or which did not show sufficient siRNA knockdown could be discarded. After DNAse treatment RNA samples were analysed on a gel for quality by assessing the prescence of 28s and 18s rRNA bands (indicating little degradation) and on the Nanodrop (Nanodrop Technologies, USA) for concentration to ensure the RNA isolation process had been successful and the samples all contained a similar level of RNA. Any samples which did not meet the criteria were discarded.

2.2.5.3 Pooling of mRNA samples

Three samples of the same treatment were pooled together to give a total starting RNA concentration of 10µg. Samples containing similar quantities as measured by the Nanodrop were pooled together to try and minimize the influence of a single sample. The volume was adjusted with DEPC water to give a total volume of 7µl. The pooled RNAs sample were placed in a PCR tube, all together there was 18 samples.

2.2.5.4 One-Cycle cDNA Synthesis

2.2.5.4.1 Poly-A RNA Control Addition

This step uses the Eukaryotic Poly-A RNA control kit (Affymetrix, USA). The Poly-A RNA control stock was diluted serially in the Poly-A Control dilution buffer following the dilutions prescribed for a 10μg starting amount of total RNA. Non-stick RNAse free microcentrifuge tubes were used for the dilutions. The first dilution was a 1:20 dilution (2μl of stock into 38μl of buffer). 2μl of this was then diluted in 98μl of buffer to give a 1:50 dilution, and finally 10μl of the resultant solution was diluted 1:5 in 40μl of buffer. Between each dilution the tube was vortexed to mix and spun down. 2μl of the final solution was spiked into each pooled sample.

2.2.5.4.2 First-Strand cDNA Synthesis

The One-Cycle cDNA Synthesis kit (Affymetrix, USA) was used for this step. 2μl of T7-Oligo(dT) primer was added to each pooled sample to give a total volume of 11μl. The samples were flicked to mix and then the contents spun to the bottom (Labnet International Inc, USA). Samples were placed in a PCR machine set to 70° for 10 minutes, they were then plunged into ice for 2 minutes. During this time a first-strand master mix was prepared: 4μl of 5x 1st strand master mix, 2μl of 0.1M DTT and 1μl of dNTP 10mM were placed in a tube per sample (x 18.1). 7μl of the master mix was added per pooled sample, the samples mixed by flicking and the contents centrifuged briefly to collect their contents to the bottom. The samples were placed in a PCR machine (PTC-200, MJ Research, USA) set to 42°C for 2 minutes, then removed and 2μl of SuperScript II added. The samples were returned to the PCR machine and incubated at 42°C for one hour. The samples were then placed on ice and cooled to 4°C. this was followed immediately by the second strand cDNA synthesis.

2.2.5.4.3 Second Strand cDNA Synthesis

Immediately following the first strand synthesis the second strand cDNA synthesis was performed. The One-Cycle cDNA synthesis kit (Affymetrix, USA) was also used for this step. A second strand master mix was prepared by mixing together for each tube: 91μl RNase free water, 30μl of 5x 2nd strand reaction mix, 3μl of dNTP (10mM), 1μl of *E.coli* DNA ligase, 4μl of *E.coli* DNA polymerase I and 1μl of RNase H. 130μl of this master mix was added to sample. This gave a total volume in the PCR tube of 150μl.

The tubes were flicked to mix and then centrifuged to collect their contents to the bottom (Labnet International Inc, USA). Then they were placed in a PCR machine (PTC-200, MJ Research, USA) programmed to do 16°C for 2 hours, followed by a 4°C hold, during which time 2µl of T4 DNA Polymerase was added to each tube. Then the samples were returned to the PCR machine and incubated at 16° for 5 minutes, followed by a 4°C hold. Then 10µl of 0.5M EDTA was added to each tube. Samples were immediately cleaned as detailed in the next section.

2.2.5.4.4 Cleanup of Double-Stranded cDNA

The Sample Cleanup module was used for this step (Affymetrix, USA). 24ml of 100% RNAse free Ethanol (BioLab Australia Ltd) was first added to the supplied cDNA wash buffer. All steps of this protocol were carried out at room temperature as specified. Firstly cDNA was transferred to 1.5ml microcentrifuge tubes. 600µl of cDNA binding buffer was added to each tube and mixed by vortexing. The colour of the mixture was checked to ensure it was yellow and not too alkaline (orange or violet). 500µl of the mixture was loaded on to a cDNA Cleanup spin column sitting in a 2ml collection tube and centrifuged for 1 minute at 10,000 x g (Biofuge Pico Heraeus, Germany). The flowthrough was discarded. The column was then reloaded with the remainder of the mixture and centrifuged again. The column was then transferred to a new 2ml collection tube and 750µl of the cDNA wash buffer applied to the column. This was centrifuged as above and the flow-through discarded. The caps on the columns were then opened and then spun for 5 minutes at maximum speed. The columns were then transferred to a 1.5ml collection tube and 14µl of RNase free water applied to the column. This was supposed to be cDNA elution buffer. The columns were incubated for 1 minute and then centrifuged for 1 minute at maximum speed. The procedure may be stopped at this point. The cDNA was stored in a fridge at 4°C overnight.

2.2.5.4.5 Synthesis of Biotin-Labeled cRNA

The GeneChip IVT Labeling kit was used for this step (Affymetrix, USA). 6µl of double-stranded cDNA was added to a new microcentrifuge tube. A master mix was prepared per sample (x18) at room temperature (to avoid precipitation of the cDNA in the presence of spermadine)of : 14µl of RNase free water, 4µl of 10x IVT labeling buffer, 12µl of IVT labeling NTP mix, and 4µl of IVT labeling enzyme mix. 20µl of

the master mix was added to each tube and mixed by flicking. The tubes were centrifuged briefly and then incubated overnight (16 hours) at 37°C in an oven incubator (Sanyo).

2.2.5.4.6 Cleanup and Quantification of Biotin-Labeled cRNA

The Sample Cleanup kit (Affymetrix, USA) was used for this step. Before beginning 20ml of 100% ethanol (BioLab Australia Ltd) was added to the IVT cRNA wash buffer concentrate to make the working solution. All steps were carried out at room temperature. 60µl of RNase free water was added to the IVT reaction and mixed by vortexing for 3 seconds. 350µl of IVT cRNA binding buffer was then added to the sample and mixed by vortexing for 3 seconds. 250µl of 100% ethanol was added to each sample and mixed by pipetting. The entire sample (700µl) was then applied to a IVT cRNA cleanup spin column sitting in a 2ml collection tube. This was centrifuged for 15 seconds at 10,000 x g (Biofuge pico Heraeus, Germany). The flow through and collection tube were discarded and then the spin column transferred to a new collection tube. 500µl of IVT wash buffer was applied to the spin column and it was centrifuged again as before, discarding the flow-through. 500µl of 80% ethanol (BioLab Australia Ltd) was applied to the spin column and then it was centrifuged again and the flow-through discarded. The caps were removed on the spin column and they were centrifuged at maximum speed for 5 minutes to allow drying of the membrane. The flow-through and collection tubes were discarded and the spin column transferred to a new 1.5ml collection tube. RNA was eluted by pipetting 10µl of RNase free water directly on to the column membrane and centrifuging at maximum speed for 1 minute. This step was repeated using 10µl of RNase free water.

The RNA concentration was then checked by measuring RNA absorbance at 260nm and 280nm on the Nanodrop (Nanodrop technologies, USA). Pure RNA should have a ratio of A_{260}/A_{280} of 2.0. For quantification of cRNA an adjusted cRNA yield had to be calculated to reflect carryover of unlabelled total RNA. Using an estimate of 100% carryover:

$$\text{Adjusted cRNA yield} = \text{RNA}_m - (\text{total RNA}_i)(y) \quad \text{Equation 6}$$

Where: RNA_m = the amount of cRNA measured after IVT (μg)

Total RNA_i=the starting amount of total RNA (μg)

y=the fraction of cDNA reaction used in IVT

cRNA samples were stored at -80°C until fragmentation.

2.2.5.4.7 Fragmenting the cRNA samples

Fragmentation of cRNA target is critical for obtaining optimal assay sensitivity. The Sample Cleanup module was used for this step (Affymetrix, USA). 20μg of cRNA (the volume of which was calculated using the adjusted cRNA concentration) was transferred to a new RNase free microcentrifuge tube. 8μl of fragmentation buffer was added along with RNase free water to give a final volume of 40μl. This was incubated at 95°C for 35 minutes then placed on ice. 2μl was removed and run on a gel to check fragmentation. The remainder of the cRNA samples were stored at -20°C. The fragmentation procedure should produce a distribution of RNA fragment sizes from 35 to 200 bases.

2.2.5.5 Eukaryotic Target Hybridisation

2.2.5.5.1 Preparation of the Hybridisation Cocktail

The 12x MES stock buffer was prepared as detailed in section 2.1.3.5 using MES hydrate (Sigma-Aldrich) and MES Sodium Salt (Sigma-Aldrich). 4.15mls of the 12x mES stock buffer was used to prepare the 2x hybridisation buffer along with 8.85mls 5M NaCl (BioLab scientific Ltd, Australia), 2.0mls 0.5M EDTA Disodium salt (Gibco, Invitrogen life technologies), 0.05ml 10% Tween 20 (BioRad Laboratories, USA) and 9.95mls DEPC H_2O.

The following was pipetted into an RNase free microcentrifuge tube: 15μg of the fragmented cRNA (30μl), 5μl of Control Oligo B2 (GeneChip® Hybridisation Control Kit Affymetrix, USA), 15μl of 20x eukaryotic hybridisation controls (GeneChip® Hybridisation Control Kit Affymetrix, USA), 3μl of 10mg/ml herring sperm (Promega Corporation, USA), 3μl of Bovine Serum Albumin 50mg/ml (Invitrogen life technologies), 150μl of 2x hybridisation buffer, 30μl of DMSO (Sigma-Aldrich) and 64μl of RNase free water to give a total volume of 300μl. The cocktail for each array was heated to 95°C for five minutes in a heat block (epindorf, Germany) and then the hybridisation cocktail samples for each array were placed on ice and driven to Liam

Williams at Auckland University's Affymetrix facility. The samples were stored at -70°C until hybridisation was carried out according to the Affymetrix protocol in an Affymetrix hybridisation oven 540 for 16 hours at 45°C. Each pooled and labelled sample was hybridised to a GeneChip® Mouse Genome 430 2.0 array (Affymetrix, USA)

2.2.5.6 Washing and Scanning of Arrays

Washing and scanning of the arrays was carried out by Liam Williams at the University of Auckland according to the manufacturer's instructions.

2.2.5.6.1 Washing and Staining of Arrays in the Fluidics Station.

An Affymetrix Fluidics station was used which was automated through use of the associated GeneChip® Operating Software version 1.4 (GCOS; Affymetrix Inc, 2005). Briefly : after hybridisation the arrays were washed with 10 cycles using Wash Buffer A (a non-stringent wash buffer) at 25°C, then 4 cycles using Wash Buffer B (stringent wash buffer) at 50°C. The array was then stained with a solution containing Streptavidin Phycoerythrin (SAPE) for 10 minutes at 25°C. This is a fluorescent molecule which binds to biotin. This was followed with 10 wash cycles using Wash Buffer A. Then a second stain was performed for 10 minutes at 25°C with an antibody solution containing Goat IgG Anti-strepavidin and biotinylated antibody to give a signal amplification effect. A third SAPE stain was applied for 10 minutes at 25°C before a final 15 cycle wash with Wash Buffer A at 30°C, a diagram of the stain is shown in

Figure 2.2-1: Staining of the biotin labelled RNA in an Affymetrix array. SAPE is a fluorescent dye which binds to biotin. The staining includes an amplification step which uses antibodies. After washing and staining the arrays were ejected from the fluidics station and transferred to a scanner.

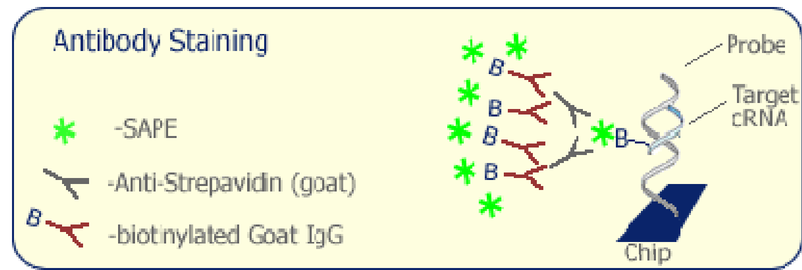


Figure 2.2-1: Staining of the biotin labelled RNA in an Affymetrix array. SAPE is a fluorescent dye which binds to biotin. The staining includes an amplification step which uses antibodies (Source: Oregon Health and Science University, http://www.ohsu.edu/gmsr/amc/amc_technology.html).

2.2.5.6.2 Probe Array Scan

Arrays were scanned using an automated system run by GCOS software v 1.4 (Afyymetrix Inc. 2005). Arrays were scanned in an Affymetrix GeneChip Scanner using a pixel value of 3µm and a wavelength 570nm. The laser scans the array and the image file data is stored as a .dat file in the GCOS software.

2.2.5.7 Microarray Analysis

The raw image file data (.dat file) generated by the scanner for each array was then automatically converted by the GCOS software to a cell intensity file (.cel file). GCOS uses the Cell Analysis Algorithm to compute a single intensity value for each probe cell on the array from the primary image file and saves this as the cell intensity file.

From this stage on analysis was carried out with the assistance of Harold Henderson and David Baird statisticians who work at AgResearch. Raw intensity data was analysed by GenStat basically following the procedure given in the GenStat manual for analysis of microarray data, pages 59 to 62 (Baird, 2006).

2.2.5.7.1 GenStat Analysis

The Cell Intensity file was imported into the GenStat software program (9th Edition, VSN International Ltd, UK) along with the mouse 430_2.CDF file. The CDF file provides information to GenStat about the location of each of the probes, and what gene each probe corresponds to. An algorithm was then applied to the data called the robust means analysis (RMA) algorithm. RMA involves three steps: background correction to remove an error component in the intensity readings; quantile

normalisation where each side is normalised to have the same cumulative frequency distribution; and summarisation where the median value for each probe set is calculated. The result is a log base 2 expression value for every gene on every slide (Baird, 2006). The expression value given is the log base 2 of the average intensity. This file was saved as 'Expressions.GSH'.

A spreadsheet file was then created which told GenStat the structure of the targets applied to the slides; that is it related the name of the .cel file (the slide name) to the treatment of the sample applied to that slide. This file was saved as 'Hybfiles.GSH'.

A spreadsheet describing the contrasts in a matrix was also prepared. This sheet described the treatment and the relevant control it needed to be compared to; it was called 'Contrasts.GSH'. The contrasts explored and the matrix used is shown below.

Table 2.2-2: Matrix showing the contrasts applied in Genstat.

Rows	Normal	TS + activin	TS + F4H	TS Only	si negative	337	773
TSA vs N	-1	1	0	0	0	0	0
TSF vs N	-1	0	1	0	0	0	0
TS vs N	-1	0	0	1	0	0	0
337 vs Si-	0	0	0	0	-1	1	0
773 vs Si-	0	0	0	0	-1	0	1
N vs Si-	1	0	0	0	-1	0	0

To estimate the differences between treatments and the relevant control a single channel ANOVA was performed on the data (since the arrays were single colour arrays). The sheets loaded were Expression.GSH, Hybfiles.GSH and Contrasts.GSH. This produced a spreadsheet which had the mean log_2 expression of each gene/probe set for each treatment, the contrast log_2 expression for each probe (simply the mean log_2 expression of a treatment – the mean log_2 expression of the control) and a *p*-value for the contrast. These results were stored in Excel. There was 45,101 different probe sets in total.

2.2.5.7.2 Identification of Genes of interest

Using Excel (Microsoft Corporation, 2003) fold change cut-offs were used to filter the log_2 contrast values to find a set of probes which was differently expressed between two treatments. The fold change cut off was chosen to obtain a target set which was

not too stringent so that genes that were truly changed would not be discarded, however the number of 'interest genes' also had to be of a manageable size. The contrasts and their fold-change cut offs are given in Table 2.2-3. The list of probes and their accompanying expression data/*p*-values for all contrasts was combined into a new spreadsheet and then any duplicate probe sets removed.

Finally a set of filters were applied to search for intersections, i.e. to find genes which were changed when more than one contrast was applied for example the set of genes which changed when 773 AND 337 siRNA treatments were done as compared to the siRNA negative control. For the siRNA experimental data probe sets which had a greater than 1.5 fold change between the siRNA negative control treated samples and the 'normal' treated samples were discarded. The *p-value* was also evaluated to ensure that probe sets which did meet the fold change cut off for a contrast also had a *p*-value of less than or equal to 0.05.

Table 2.2-3: Contrasts and foldchange cut-offs used in obtaining a target set of interest genes

Contrast	**Fold Change Cut-Off**
TS medium without growth factors vs N (Normal 70%CM + F4H)	2
TS medium + Activin vs N (Normal 70%CM + F4H)	2
TS medium + Fgf4 vs N (Normal 70%CM + F4H)	2
337 siRNA vs siRNA negative control	1.4
773 siRNA vs siRNA negative control	1.5
337 siRNA vs N (Normal 70%CM + F4H)	1.5
773 siRNA vs N (Normal 70%CM + F4H)	1.5

2.2.5.7.3 Annotation and Verification of Interest Genes

Probe sets from the intersections of contrasts were then matched to gene names using Ingenuity Pathway Analysis software 4.0 Version 3. Ingenuity is a software package which uses peer reviewed scientific journals (approximately 400 journals) to give information about gene product interactions and pathways, functional analysis of data and gene ontology. Information on each probe set for each intersection studied was tabulated to give summary gene ontology for each probe set identified. Information included canonical pathway, molecular function and biological process.

Using this information a subset of genes was chosen which were of interest. Real-time PCR primers were designed for these and then the expression of these genes was analysed by quantitative real-time PCR to check their expression was truly significantly different (section 2.2.3). The cDNA used for the verification was derived from the same samples used for microarray analysis.

CHAPTER 3 : siRNA INDUCED *Elf5* KNOCKDOWN

3.1 OVERVIEW

Targeted *Elf5* mRNA degradation using siRNA was used to modulate Elf5 expression as a means of identifying Elf5 target genes. *Elf5* knockdown was achieved using two 25-mer siRNA oligonucleotides and an optimised protocol was established. Measurement of *Elf5* knockdown and other candidate genes known to be involved in TS cell differentiation was achieved using real-time PCR.

3.1.1 RNA Interference

RNA interference or RNAi was discovered by Craig Mello and Andrew Fire in 1998 when they observed that double stranded RNA could induce a sequence specific silencing response in the nematode worm *Caenorhabditis elegans* (Fire *et al.*, 1998). Not long after double stranded RNA (dsRNA) was shown to also work in mammals when it was used to inhibit the function of three genes in the mouse embryo (Wianny & Zernicka-Goetz, 2000). Double stranded RNA has been found to cause silencing through targeted degradation at the mRNA level (Cullen, 2005). Since the discovery of dsRNA interference a greater understanding into the mechanisms and essential characteristics of successful RNAi molecules has allowed gene specific design and the reduction in non-specific effects, so that almost any gene can be selectively targeted with high success. One of the important discoveries has been that a sequence of just 21-25 nucleotides in length (small interfering RNA; siRNA) is able to elicit a gene specific response.

RNAi experiments to reduce gene expression are advantageous over classic knockout experiments because they take a much shorter time to achieve success. Commercial companies such as Invitrogen Life Technologies can quickly design and manufacture RNAi molecules so that successful gene knockdown may be achieved within weeks, rather than laborious gene targeting experiments which take months (Weber *et al.*, 1999). A disadvantage of RNAi experiments is that they can elicit an interferon response, normally reserved for viral infections (Bridge *et al.*, 2003) and therefore cause changes in gene expression other than the target. Also, due to the high tolerance for mismatches between the 2-8 nucleotide 'seed' region of a siRNA other genes than the

target may be affected in so called off target effects (Echeverri *et al.*, 2006). To ensure responses are specific for a given target sequence, and result in a reduction in transcript number ("knock-down") it is recommended that multiple siRNAs targeting different regions of the same gene be used. If they give the same phenotype then it is very unlikely to be an off-target effect (Echeverri *et al.*, 2006). The efficiency of knockdown can also greatly depend on the location of the targeted sequence within the mRNA (Li *et al.*, 2006).

A disadvantage of RNAi experiments is that the effect of RNAi induced knockdown is only transient. Transfection with siRNA is thought to have an effect for up to five days following transfection (Dykxhoorn *et al.*, 2003); however longer lasting gene inactivation of up to 13 days has been reported (Li *et al.*, 2006).

Four Elf5 mRNA targeted siRNA molecules were designed (Section 3.2.1) using the Invitrogen website (Invitrogen Life Technologies; www.invitrogen.com). These 'stealth' siRNAs have a chemically modified RNA back bone which is supposed to reduce non-specific effects and minimise the chance of an interferon response (Invitrogen Life Technologies). *Elf5* mRNA knockdown was quantified using reverse-transcription (RT) real time PCR.

3.1.2 Real Time PCR

The siRNA knockdown of *Elf5* and the subsequent changes in gene expression were analysed using the method of real-time RT-PCR (reverse transcription-polymerase chain reaction). Real time PCR measures the level of DNA which has been amplified after each cycle of a PCR, hence the term 'real time'. This is achieved through the detection of a number of fluorescent probes which are directly correlated to the amount of amplified product. A widely used fluorescent probe is SYBER-Green. SYBER-Green intercalates into the DNA helix giving an 11 fold greater fluorescence signal in double stranded DNA then in single stranded DNA at 530nm (Zipper *et al.*, 2004); therefore SYBER-Green fluorescence is directly correlated to the level of double stranded DNA. Because SYBER-Green measurement does not depend on the sequence of template/primers the same dye can be used for different reactions. A downside of this is that other double stranded DNA molecules may be present and also contribute to fluorescence, such as primer-dimers. To ascertain the contribution of

primer duplexes to fluorescence the melt curve for each reaction was checked. Primer-dimers have a low melting point, therefore a DNA product melt curve with a low melting point or multiple melting peaks is indicative of primer-dimers. Primers were designed using software which minimises complementarity between primers and prevent primer dimmers (section 2.1.3.6).

The initial starting level of a template is estimated by the cycle at which fluorescence of the product crosses a threshold; the more templates at the beginning of the reaction the lower the number of cycles it takes to cross the threshold. The 'threshold' cycle (Ct) is the cycle at which the level of fluorescence is significantly higher than background fluorescence and the reaction is in the early exponential phase, with maximum amplification efficiency (Wong & Medrano, 2005). At this phase in a real-time PCR reaction the amplification efficiency is constant and is not limited by reagents. As the reaction proceeds, reagents become limiting and the amplification efficiency is reduced (the plateau phase) (Wong & Medrano, 2005). In the ABI machine the cycle at which fluorescence crosses the threshold level is called the Ct (Applied Biosystems, USA). In the Rotor-gene 6000 the 'takeoff' point can be used:

"The second derivative of the amplification plot produces peaks corresponding to the maximum rate of fluorescence increase in the reaction. The Takeoff point is defined as the cycle at which the second derivative is at 20% of the maximum level, and indicates the end of the noise and the transition into the exponential phase" (Rotor-Gene 6000 Series Software version 1.7.61, help menu).

Real time PCR assays are 1000 times more sensitive than dot-blots (Wong & Medrano, 2005) and can detect small variances in gene expression; as little as a 25% gene expression difference can be detected between samples, provided enough replicates are done (Gentle *et al.*, 2001). This is important in the context of this experiment, because transfection efficiency varies among cells; different cells will have different degrees of *Elf5* knockdown or over-expression. However all cells are harvested and their RNA extracted for use in gene expression measurements. This means changes in gene expression as a result of Elf5 modulation will be averaged across the cells, and cells with very low transfection efficiency will muffle the effect, so a sensitive technique needs to be utilised for gene expression.

A two step RT-PCR method was chosen, where cDNA synthesis and PCR amplification were carried out separately. After reverse transcription cDNA was diluted

at least 1:20 in PCR grade water. This method was chosen because it is known that reagents in the reverse transcription such as DDT and reverse transcription can inhibit the reaction (Liss, 2002). Furthermore, by diluting the cDNA 1:20 many different genes can be assayed using one cDNA sample, so reference gene assays do not need to be carried out as often. A DNase step was included in the procedure directly after TRIZOL extraction because DNA contamination can make up to 80% of purified nucleic acid and therefore changes in DNA contamination may be interpreted as changes in gene expression (Bustin, 2002).

3.1.2.1 Normalisation of RT-PCR

The procedure from cell harvesting and RNA isolation through to RT-PCR involves a number of steps (section 2.2.3), all of which can be variable between samples (for example starting material, cell transcriptional activity, RNA isolation efficiency). A particular source of variation is the efficiency of reverse transcription which can range from 5% to 90% and is influenced by salts, alcohols or phenols remaining from RNA isolation giving inter-tube and inter-experimental variability (Freeman *et al.*, 1999). To allow for comparison between samples the variation between genes and samples needs to be normalized using a control. The sequence and secondary structure of mRNA also influences RT efficiency; this can cause the enzyme to stop and dissociate from the mRNA (Bustin, 2002). To minimize these effects primers were chosen which amplified the 3' end of a gene, which is the first part to be reverse-transcribed when using an oligo dT primer.

Normalisation can be done using a number of controls; addition of synthetic RNA (or later DNA), total RNA or ribosomal RNA. However all of these have their drawbacks such as not being present in all the steps and therefore not controlling for variation in previous steps or that total RNA and rRNA measurements can bear little correlation to mRNA levels since mRNA makes up only a small percentage (~7.5%) of total RNA and this proportion can vary with transcriptional activity (Vandesompele *et al.*, 2002). Ribosomal RNA is also not suitable as a control in this experiment since it is not reverse transcribed using an oligo dT primer, and therefore cannot be measured using RT-PCR. An internal control gene does not have any of these problems since it is itself an expressed gene and is therefore treated identical to target genes. However it is important that the internal control gene does not vary with the treatment because then

it could increase the significance of a result (if it had the opposite effect of the target gene upon treatment) or it could reduce the significance of a result (if it had the same effect as the target gene from treatment). It has been reported in a number of papers that supposed 'housekeeping genes' can change during the differentiation of stem cells (Hamalainen *et al.*, 2001; Willems *et al.*, 2006). The resounding advice is to screen a myriad of housekeeping genes until a suitable candidate is chosen to use in normalization calculations which does not vary with treatment. (Hamalainen et al., 2001; Livak & Schmittgen, 2001; Perkel, 2006; Pfaffl *et al.*, 2004; Willems et al., 2006; Wong & Medrano, 2005), or to use multiple housekeeping genes, and use the geometric mean of these (Vandesompele et al., 2002).

3.1.2.1.1 Analysis of RT-PCR Data Generated by the ABI 7700

Relative quantification was used to calculate the changes in gene expression for a sample compared to an untreated control. The calculations used were based on the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The difference in Cts between the target gene and the normalizing gene is first calculated ($\Delta Ct_{treatment}$) for a treated sample and the control sample ($\Delta Ct_{control}$) to normalize the expression. The difference between the treated normalized sample and the control sample is then calculated ($\Delta Ct_{treatment}$-$\Delta Ct_{control}$). This is then converted into initial expression of the target compared to the control by using the equation:

Relative Target expression $=E^{-(\Delta Cttreatment-\Delta Ctcontrol)}$ Equation 1

Where E is the amplification efficiency and using the assumption amplification efficiency is equal for the normalizing gene and the target gene.

This is then changed to the equation:

Relative Target Expression $=2^{-(\Delta Cttreatment-\Delta Ctcontrol)}$ Equation 2

Using the assumption amplification efficiency is 2.

A problem with this method of calculation is that it assumes that amplification efficiency is perfect (i.e. 2 represents a doubling every cycle), the amplification efficiency is also assumed to be equal for the target template gene and the housekeeping gene. However this is often not the case and an amplification efficiency difference of just 5% between two initially equal targets can result in one target appearing twice as abundant after 26 cycles of PCR (Freeman *et al.*, 1999). To avoid

this error, the efficiency for primer pairs was determined as follows: a standard curve was made by making a dilution series of one of the samples. The $-\log_{10}$(dilution) was plotted against the Ct. Points which were obviously not in the linear part of the curve were discarded. The efficiency of each gene reaction could then be calculated by :

$$E=10^{slope} \qquad \text{Equation 3}$$

3.1.2.1.2 Analysis of RT-PCR Data Generated by the Rotor-Gene 6000

It has been suggested that amplification efficiency calculated from raw data is more accurate than that from a standard curve (Tichopad *et al.*, 2003; Wong & Medrano, 2005). The Rotor-Gene 6000 software (v1.7, Corbett Research, Australia) uses the average increase in raw data for the 4 cycles following the Takeoff to calculate a sample's amplification. Therefore each individual reaction has associated amplification efficiency. The Takeoff, as described above, is used in an analogous way to Ct in relative expression calculations.

3.2 ATTAINMENT OF *Elf5* KNOCKDOWN

Four *Elf5* targeted siRNAs were trialled for their ability to achieve *Elf5* knockdown in TS cells when transfected using Lipofectamine 2000 (Invitrogen Life Technologies). Two of these were successful and used to establish a standard protocol to achieve greater than 80% *Elf5* knockdown. Knockdown of a gene using RNAi can also induce an interferon response (Bridge *et al.*, 2003). The induction of two viral responsive Interferon genes were measured in 337 siRNA treated cells compared to un-transfected TS cells to ensure an interferon response was not being activated.

3.2.1 Design of Stealth siRNAs

Four siRNAs were designed using the reference sequence for Elf5; NM_010125.
The sequences of the Elf5 targeted siRNA oligonucleotides were designed using the BLOCK iT™ siRNA designer available at:
https://rnaidesigner.invitrogen.com/sirna/setOption.do?designOption=stealthit
(Invitrogen Life Technologies). Several possible 25-mer siRNAs were returned ranked in order of predicted knockdown efficiency. The top four sequences were selected and Stealth™ synthetic RNA of their sequences ordered from Invitrogen Life Technology.

Stealth™ siRNA is chemically modified so to give greater stability, less cellular toxicity and higher specificity (www.invitrogen.com). The siRNAs were designated names based on the starting position of the Elf5 mRNA to which they are targeted. The names and rating of the siRNAs are given below.

Table 3.2-1: The top four results from the Block it siRNA designer. Each siRNA is named based on the start of its corresponding targeting sequence in the Elf5 mRNA.

Start Position	Sense RNA Sequence 5'-3'	GC%	Rank (out of 5)
337	CCAACUGCAUCUCCUUCUGUCACUU	48	5 stars
811	GGGUUGACCGGAGGUUAGUGUACAA	52	5 stars
770	CCGAGCCCUGAGAUACUACUAUAAA	44	5 stars
733	GGAAGAAGAAUGACAGGAUGACGUA	44	4 stars

3.2.2 Optimisation of Elf5 Knockdown

Transfections of siRNA were carried out in $10cm^2$ well dishes following the Lipofectamine 2000 protocol for siRNA transfection (section 2.2.1.7). The day after transfection cells were harvested and total RNA extracted using TRIZOL (Invitrogen Life Technologies) followed by reverse transcription and real-time PCR to measure relative expression of Elf5 compared to a siRNA control treated sample (section 2.2.3). Expression levels were normalised to a housekeeping gene such as β-Actin before relative expression was calculated.

Two factors could influence the level of Elf5 mRNA expression knocked-down in mTS cells:

- The efficiency of the particular siRNA at knock-down the *Elf5* mRNA due to its ability to interact with the RNAi cellular machinery; in particular its ability to direct the RNA induced silencing complex (RISC) to the target mRNA. (Hannon, 2002).
- The transfection efficiency of the siRNA complex into the TS cell.

Both of these factors were optimised in the course of this work.

3.2.2.1 Trial of the *Elf5* targeted siRNAs

The Lipofectamine 2000 (Invitrogen Life Technology) protocol was followed which recommends using 100pmol of siRNA per transfection (Invitrogen life technologies, 2006). In order to measure transfection efficiency 20pmol of a fluorescently labelled

control siRNA oligo (Invitrogen Life Technologies) was also transfected into one of the wells. The relative expression of *Elf5* in each if the siRNA treated samples compared to a non-treated control well is shown below (Figure 3.2-1) as measured by real-time PCR following RNA extraction and reverse transcription (section 2.2.3).

Elf5 expression was normalised to β-Actin expression before relative expression was calculated. There were clear differences in the efficacy of *Elf5* knockdown between different siRNAs. The two *Elf5* targeted siRNA molecules which showed the greatest knockdown were 337 and 733; however un-optimised knockdown was only about 40%. These siRNAs were then used further to optimise transfection efficiency.

Surprisingly the fluorescent siRNA molecule appeared to show an increase in relative *Elf5* expression. On closer inspection it was found that the absolute *Elf5* Ct was the same as the non-treated control sample and instead the Ct of *β-Actin* had increased. This indicated that the fluorescent control siRNA was having non-specific effects on *β-Actin* and was not a suitable control. Following this experiment a stealth siRNA negative control duplex (Invitrogen Life Technologies) was used to transfect the control samples.

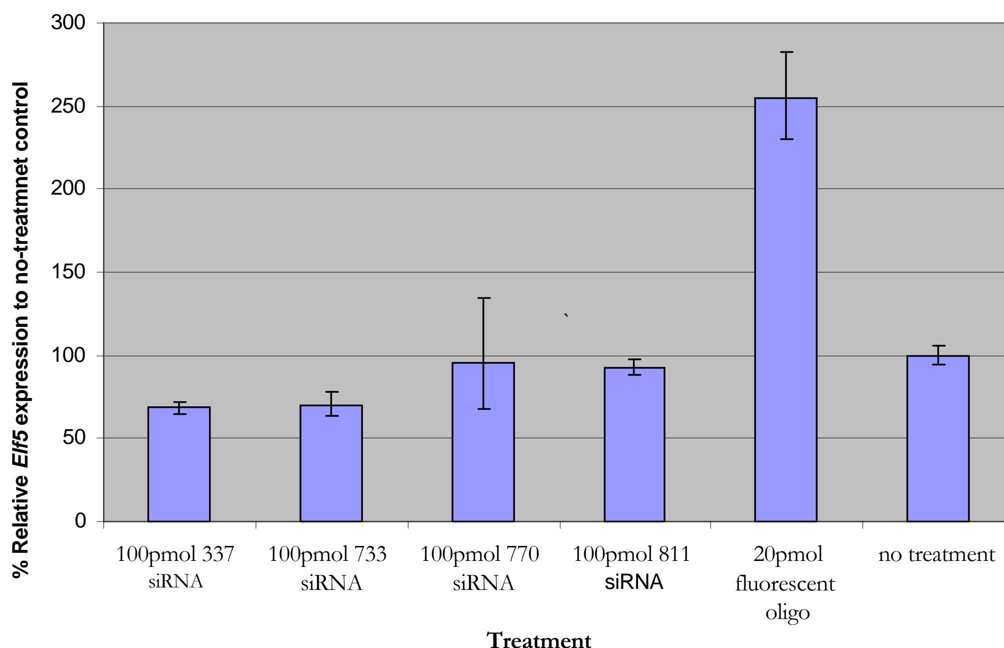


Figure 3.2-1: Trial experiment on the *Elf5* targeted siRNAs. *Elf5* expression was normalised to *β-Actin* expression before being expressed relative to the no-treatment control (the control treatment was set at 100%). The fluorescent oligo appeared to have non-specific effects on *β-Actin* expression. (Relative *Elf5* expression +/- sem, n=2).

3.2.2.2 Transfection Optimisation

3.2.2.2.1 Optimal Seeding Density

The cationic lipid transfection reagent, Lipofectamine (Invitrogen Life Technology) was used to transfect siRNA into mTS cells. The main factor influencing the transfection efficiency of cells when using cationic lipid solutions, is the ability to transfect cells in a healthy and growing state (Ausubel *et al.*, 1999). Therefore a seeding protocol needs to be established which allows cells to be exponentially growing at the time of transfection. The manufacturers recommend a cell confluency of 30% at transfection (Invitrogen Life Technologies). By trial and error an optimal seeding density protocol was established to enable cells to be at the correct confluency at the time of transfection (section 2.2.1.7.1). Cells were seeded the day before a planned transfection to allow them to recover and be proliferating at the time of transfection.

3.2.2.2.2 Optimal siRNA Concentration

The Lipofectamine 2000 protocol suggests altering the final concentration of transfected siRNA to optimise transfection efficiency (Invitrogen life technologies, 2006). Three amounts of the best siRNA (337) were tested (100, 150 and 200pmol). Two amounts of 733 siRNA were tested (100 and 200pmol). Included in the experiment was the effect of using a combination of the two best siRNA oligos to see if *Elf5* knockdown could be improved. The results from this set of experiments are shown in Figure 3.2-2. The 337 siRNA when transfected at 100pmol gave an average of 95% knockdown of Elf5 expression relative to the 100pmol control. Further increases in the amount of siRNA transfection only reduced the relative knockdown. The 733 siRNA showed a smaller knockdown efficiency.

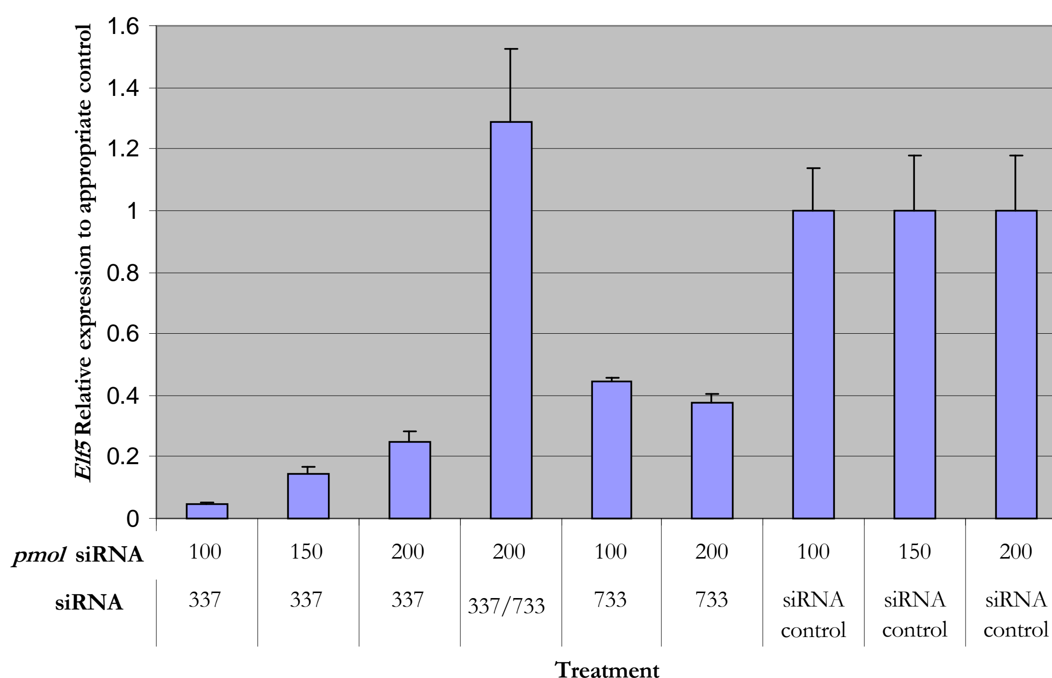


Figure 3.2-2: Optimisation of *Elf5* knockdown by altering siRNA concentration. *Elf5* expression was normalised to *β-Actin* expression and is given relative to the appropriate control transfected with the same amount of siRNA control oligonucleotide. The average control normalised expression was set at 1. Bars show relative Elf5 expression +/- sem, n=3.

3.2.2.3 Estimation of Transfection Efficiency

Once the optimal efficiency had been achieved as measured by Elf5 knockdown, to allow an estimate of transfection efficiency, the siRNA fluorescent oligo (Invitrogen Life Technologies) was transfected into TS cells following the optimised protocol. Unfortunately 100pmol of fluorescent oligonucleotide was unable to give a strong enough signal (photo not shown) to be detected by fluorescent microscopy (section 2.2.1.11). The fluorescent labelled siRNA had to be transfected at 240pmol for a clear signal to be seen. Although this deviated from the optimised protocol it still gives some indication of transfection efficiency. The fluorescent siRNA oligo is designed to localise primarily to the nucleus. Figure 3.2-3 shows a merged bright field and fluorescent photo taken of TS cells using a *10x* objective. Fluorescence was mainly concentrated in the nucleus of cells; however, a high proportion of cells showed a green hue indicating high transfection efficiency. At least some fluorescence was estimated to be seen in 80-90% of cells.

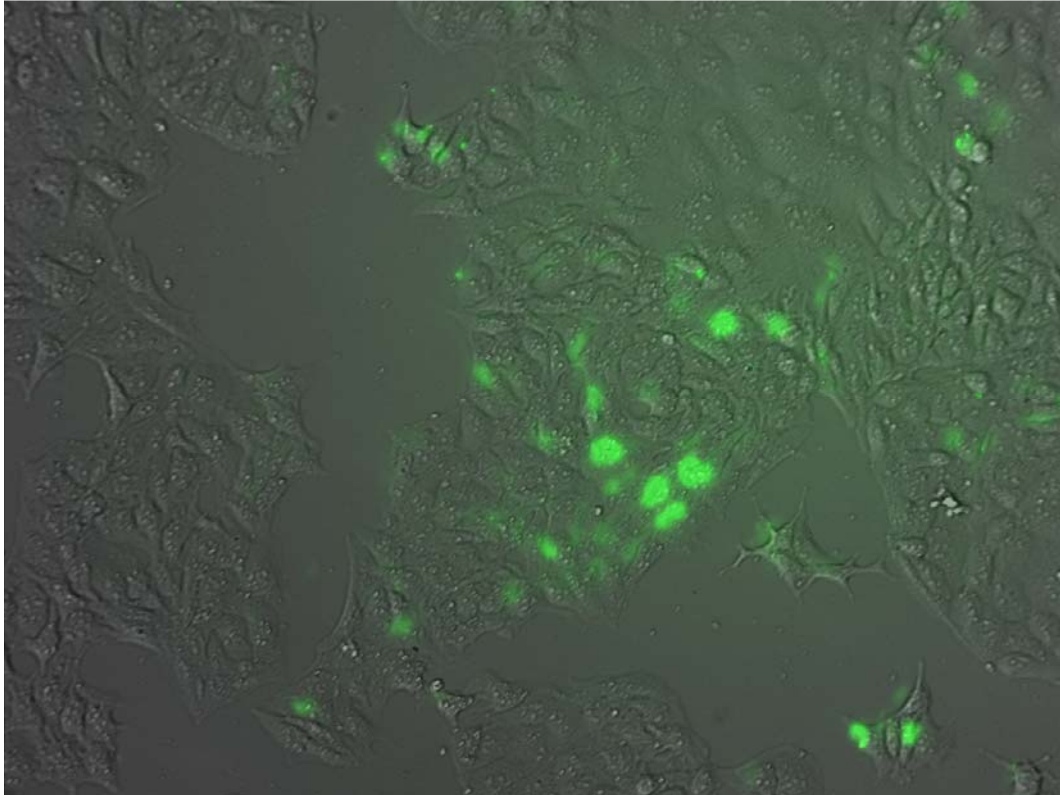


Figure 3.2-3: *x10* bright field and fluorescent merged photo of TS cells transfected with 240pmol of fluorescent oligonucleotide. Fluorescence has been artificially coloured green.

3.2.3 Measurement of an siRNA Induced Interferon Response

To ensure an interferon response was not being initiated by treatment of TS cells with siRNA, the expression of the genes Interferon α-*4* (*Ifna4*) and Interferon-β (*Ifnβ*) were measured. Both of these genes are members of the type I interferons, which are induced by viral infection (Samuel). Primers were designed using the mRNA reference sequence for these genes (2.1.3.6). The Interferon genes did not contain any introns. To ensure the primer pairs worked the primers were used in a PCR reaction (section 2.2.2.9) with mouse genomic DNA. Each primer set gave a product of the correct size as indicated by running the PCR products on an agarose gel (not shown). An aliquot of the PCR product for each primer pair was used as a positive control for real-time PCR analysis. Figure 3.2-4 shows the results of the relative expression of both Ifnα4 and Ifnβ in 337 siRNA treated TS cells and non-transfected control cells. There was no significant difference in expression between siRNA treated cells for Ifnα4 ($P=0.60$) or Ifnβ ($P=0.46$).

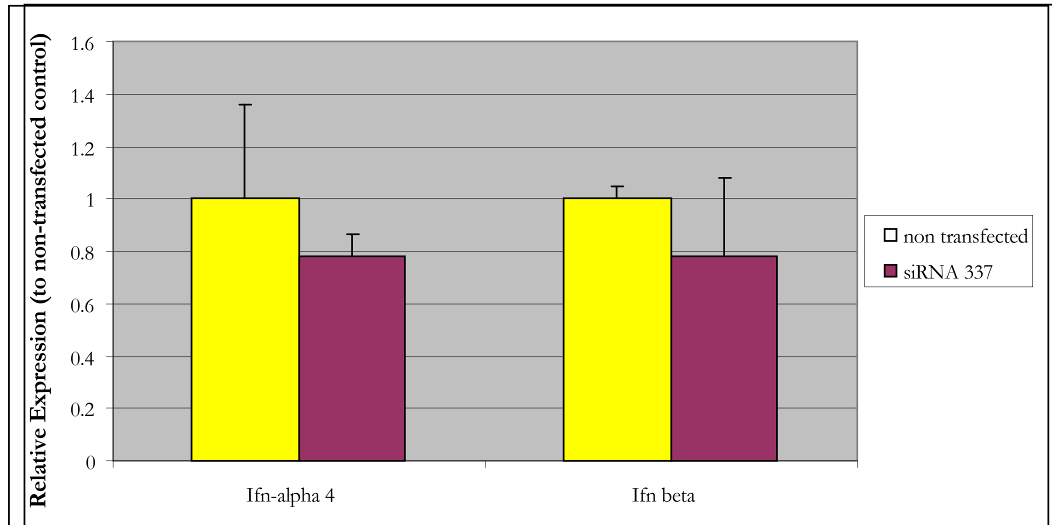


Figure 3.2-4: Relative Expression of Interferon-alpha4 and Interferon-beta in TS cells transfected with 337 siRNA and non-transfected controls. Interferon expression was normalised to *Gapdh* expression and expressed relative to the control (set at 1) +/- standard deviation; n=5. There was no significant difference berween siRNA treated cells with the control: Interferon-alpha 4 P=0.60; Interferon-beta P=0.46.

3.3 EFFECTS OF *Elf5* KNOCKDOWN ON TS CELLS: CANDIDATE GENE EXPRESSION AND MORPHOLOGICAL CHANGES

Subsequent to achieving an acceptable level of knockdown, the effects of reduction of *Elf5* expression in TS cells were studied. Real-time PCR analysis was done on samples successfully showing *Elf5* knockdown for selected genes reported to be involved in trophoblast maintenance and differentiation. This candidate gene approach was hoped to identify downstream target genes of Elf5 in TS cells. During the course of this work several problems were identified in the protocol used for isolation of RNA through to analysis of expression using real time PCR (2.2.3.1) to develop a new protocol (2.2.3.2).

3.3.1 Protocol changes

The original procedure given in section 2.2.3.1 was taken from an in-house lab protocol. Following siRNA transfection (2.2.1.7.3), cells were harvested with Tryple (Invitrogen Life Technologies) and then RNA extraction done with TRIZOL reagent (Invitrogen life technologies, NZ). A DNase treatment was performed, and the total RNA reverse transcribed to produce cDNA. Finally, real-time PCR was used to

measure the relative expression of cDNA transcripts. During the course of this work a number of problems were encountered with this protocol. Subsequently modifications were made to this protocol. The modifications and reasons for the changes are given for each section.

3.3.1.1 Original Results

Original real-time PCR results showed several problems:

1. **The raw Ct data for the housekeeper gene (*β-Actin*) was highly variable**

This observation could not be explained simply by different numbers of cells harvested because all wells in an experiment were seeded in an identical way, and therefore should have relatively consistent levels of RNA. However the Ct value for *β-Actin* could vary by 9 units (Table 3.3-1). Given an amplification efficiency of 2, that is a doubling every cycle, 9 Ct units corresponds to a 2^9 fold difference, i.e. greater than a 500 fold difference in the level of *β-Actin* between two samples. Although the relative level of *Elf5* was of interest, which did change as *β-Actin* changed, this problem was of concern as it indicates large variation in the efficiency of RNA extraction and/or reverse transcription between samples.

Table 3.3-1: An example of the variation in β Actin Ct which could be measured using real-time PCR following RNA harvesting and reverse transcription. The cells were all seeded at the same density so variation in cell number does not account for observed variation.

Treatment	Average β- Actin Ct	Standard deviation (n=2)
Elf5-337	33.82	0.05
siRNA negative control	24.96	0.52
Elf5-337	32.14	0.49
siRNA negative control	30.04	0.12
Flourescent Oligo	38.30	0.13
TS medium	32.70	0.02
TS medium +F4H	32.64	0.01
Normal growth medium	25.84	0.34
TS medium + activin	28.81	0.12
Normal growth medium	37.11	0.12

2. The DNase treatment was not very efficient

After RNA isolation with TRIZOL and an ethanol precipitation RNA was resuspended in 15µl of RNase free water and treated with 1µl of amplification grade DNase enzyme to remove contaminating DNA. Contaminating DNA can also be amplified during the quantitative RT-PCR reaction (especially for genes not containing introns and for which pseudogenes exist). The reaction proceeded at 37°C for 30

minutes. After this the RNA sample was purified using ethanol precipitation and resuspended in 11μl of RNase free water. The entire total RNA sample was used in the reverse transcription reaction except for 1μl, which was resuspended in 19μl of TE buffer to give the reverse transcription negative (RT-) control. Each sample analysed by real time PCR had its corresponding RT- sample analysed with a housekeeping primer set, such as *β-Actin* or *Gapdh*, to check for the level of DNA contamination. DNA contamination is indicated by the *β-Actin/Gapdh* Ct of the RT- sample being similar to the cDNA *β-Actin/Gapdh* Ct. Although some experiments had very high Cts for the RT- sample (indicating low DNA contamination), others showed definite DNA contamination, and therefore had to be discarded.

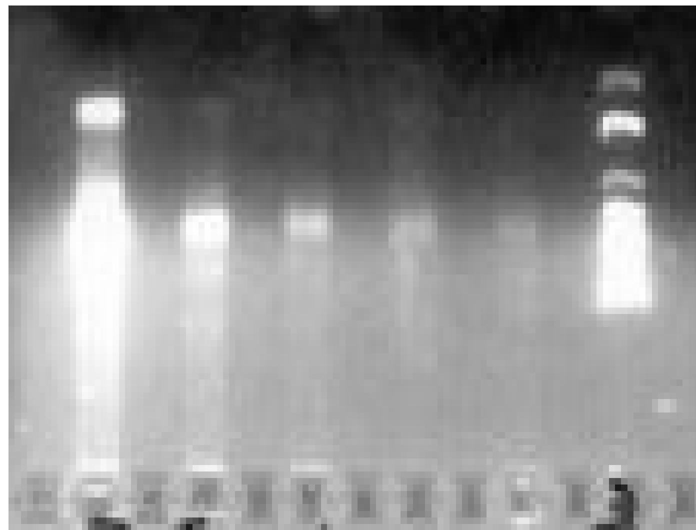
To correct for this error the amplification grade DNase I enzyme (Invitrogen Life Technologies) protocol was consulted. The protocol suggests 1μl of DNase enzyme per 1μg RNA sample. By analysing a selection of total RNA samples with the Nanodrop spectrometer (Nanodrop Technologies, USA) it was found these varied considerably in concentration, however the average sample amount was about 4μg of total RNA. The level of DNase used in each DNase reaction was increased 4-fold to 4μl, and the time of incubation increased to 1 hour. Corresponding changes in volume were made for the other reagents.

Table 3.3-2: Changing the DNase protocol reduced the level of DNA contamination. The Ct for Gapdh is shown for a cDNA and corresponding RT- sample using the original protocol of 1μl of DNAse I. The results from an experiment where 4μl of DNase I were used are also shown. The change in the amount of enzyme used greatly reduced the level of DNA contamination.

Sample Number	1μl of Dnase I for 30 minutes		4μl of Dnase I for 60 minutes	
	1/20 cDNA Gapdh Ct	1/20 Gapdh Rt-	1/20 cDNA Gapdh Ct	1/20 Gapdh Rt-
1	24.45	26.58	22.07	30.47
2	24.16	23.80	26.93	32.31
3	24.21	24.21	21.85	29.63
4	24.63	25.78	19.07	30.37
5	24.28	24.24	20.61	30.15
6	22.91	22.07	18.10	29.21
7	23.41	23.99	21.52	32.77
8	23.77	23.13	17.86	29.88
9	23.22	22.22	16.66	31.46
10	24.38	25.28	19.56	28.55
11	24.84	25.13	20.08	28.58
12	25.32	25.03	22.85	30.65

3.3.1.2 RNA degradation during the Cell harvesting

During isolation of RNA for the microarray the total RNA samples were run on a gel to check RNA quality as indicated by the clear presence of sharp ribosomal RNA bands. Unfortunately most of the isolated RNA samples showed RNA degradation/poor isolation as seen in Figure 3.3-1: RNA samples showing degradation. 1µl of isolated RNA was run on a 1.4% agarose gel to check to see that the two rRNA bands could clearly be seen. One sample was chosen at random from the 12 samples of RNA which were to be used for the microarray. The TS only sample could easily be seen, however the other samples run did not have clear rRNA bands indicating possible RNA degradation.. Upon suggestion of a colleague (Dr. David Pearton) the dispersion of the cells using the recombinant Trypsin enzyme could be a possible source of RNases. The TRIZOL information sheet was obtained from Invitrogen (Invitrogen life technologies, 2003) and this also mentioned Trypsin digestion of cells as a source of RNases. The manufacturers advise applying TRIZOL directly to cells in the culture dishes after rinsing with PBS. This advice was followed.



TS Only	TS + f4H	TS + Activin	siRNA 733	Normal medium	1kb ladder

Figure 3.3-1: RNA samples showing degradation. 1µl of isolated RNA was run on a 1.4% agarose gel to check to see that the two rRNA bands could clearly be seen. One sample was chosen at random from the 12 samples of RNA which were to be used for the microarray. The TS only sample could easily be seen, however the other samples run did not have clear rRNA bands indicating possible RNA degradation.

RNA samples extracted after this protocol change showed good quality as seen by gel analysis (Figure 5.1-1) and the raw housekeeper Ct was also a lot less variable between samples. These results indicate the recombinant Trypsin enzyme had been a likely source of RNase contamination.

3.3.2 Candidate Gene Analysis

Using a set of RNA samples which had greater than 80% Elf5 knockdown, the relative expression of a number of genes known to be involved in TS cell differentiation was analysed by real time PCR. The ABI machine was used and the amplification calculated for each gene primer set using a dilution series of one of the samples (section 2.2.3.3). The relative expression was calculated compared to a siRNA negative control set of samples. The candidate genes screened and a brief description of their role in TS cells is given in Table 1:2.2-1. A total of 16 genes were screened, however primer sets for *Ets2*, *Stra13*, *Gcm1* and *Mash2* did not give a DNA product, or if there was one it was at the limit of detection and not linear in amplification. The results from the relative expression of the primer sets are shown in Figure 3.3-2. Also included is a sample grown in medium without Fgf4 or Activin. No error bars or *p values* could be calculated for the relative expression of this sample because it was a single sample.

Table 3.3-3: Candidate genes analysed in Elf5 siRNA samples. TE= trophectoderm, EXE=extra-embryonic ectoderm, TS= trophoblast stem cell

Gene Name	Function in Trophoblast Stem Cells/Mouse Embryo
Esrrb	Required for maintenance of ExE and chorion, knockout gives over-production of giant cells
Mash2/Ascl2	Spongiotrophoblast marker, deletion results in loss of spongiotrophoblast and increase in giant cells
Hand1	Expressed in EPC and giant cells, required for giant cell formation, over-expression causes TS cells to differentiate in to giant cells
Gcm1	Required for syncytiotrophoblast formation
Tbr2/eomes	Extra-embryonic ectoderm marker, required for maintenance of proliferation
Fgfr2	Receptor for Fgf4 signalling, required for proliferation of TS cells and the ExE
Tcfap2c	Required for extra-embryonic lineages, knockouts die by E8.5, however still are able to gastrulate
Bmp4	Expressed in ExE
Ehox	Expressed in EXE, chorion and chorionic trophoblast of the labyrinth, also in TS cells
Stra13	Expressed in giant cells, required for giant cell formation, over-expression in TS cells leads to giant cell formation
Cdx2	Earliest known TE specific gene. Required for ExE formation
Spc4/Pace4	Protease secreted by the ExE, cleaves Nodal
Furin/Spc1	Protease secreted by the ExE, cleaves Nodal
Nodal	Expressed in the TE, required for up regulation of Fgf4 signalling from the epiblast
Ets2	Required for development of the extra-embryonic ectoderm

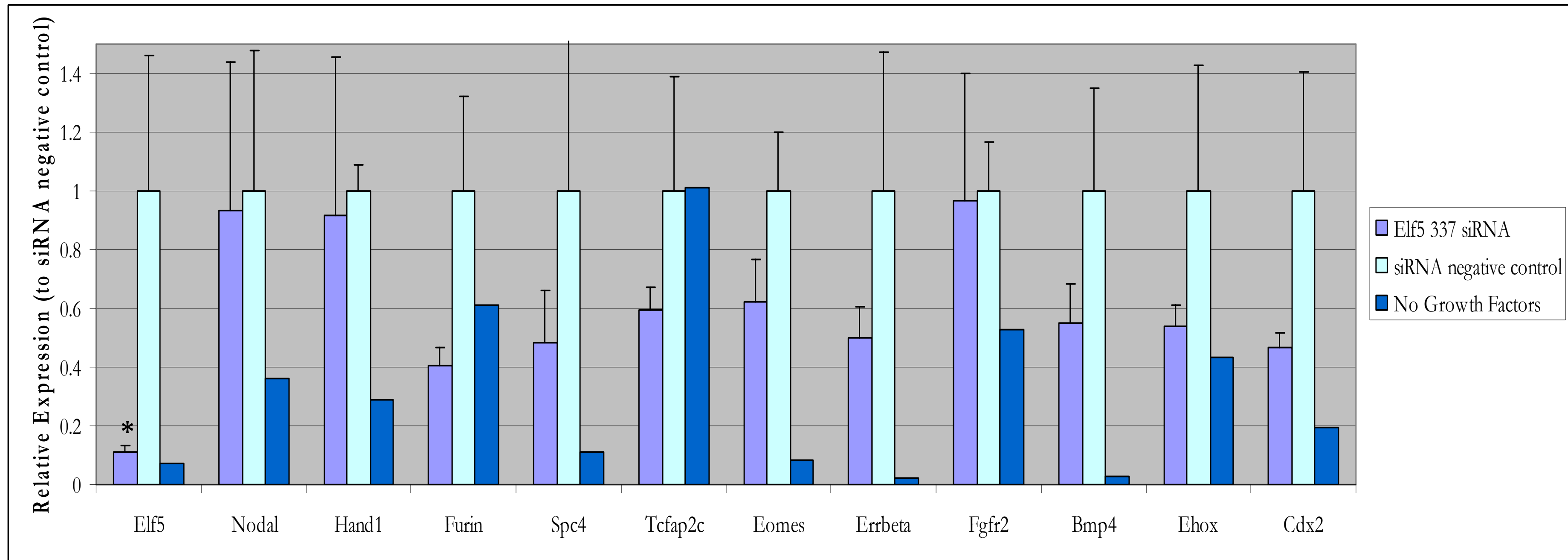


Figure 3.3-2: Relative Expression of candidate genes measured in TS cells treated with Elf5 337 siRNA. The average of three samples is shown +/- the standard deviation. The candidate gene expression of a sample deprived of Fgf4 and Activin for 24 hours is also shown, however no error bars can be shown since itt was a single sample *=significant (P<0.05)n=3 except for the no growth factor sample where n=1.

The only gene that was significant after calculation of P values using the students T test was *Elf5*with a p value of 0.002. *Eomes* and *Furin* had low *p values* just above 0.05 of 0.06 and 0.08 respectively.

3.3.3 Morphological Effects of transient Elf5 knockdown

Elf5 is known to be important for maintaining the stem cell nature of mTS cells; down-regulation of Elf5 occurs concomitantly with differentiation (Donnison *et al.*, 2005) The predominant differentiated cell type observed in cell culture is the giant cell. As the name implies giant cells are much larger than their TS cell precursors, they are flat cells with large nuclei containing many copies of the DNA due to their interesting ability to replicate their DNA without undergoing mitosis (Simmons *et al.*, 2007). The day after *Elf5* 337 siRNA transfection, mTS cells were observed to see if there was an increase in the frequency of giant cells. A sample grown for twenty four hours without Fgf4 or Activin growth factors was also analysed. No morphological changes in the cells were observed in either siRNA treated TS cells or TS cells deprived of growth factors. This observation was also confirmed by using flow cytometery (FC) to see if an increase in DNA (stained with Propidium Iodide) per cell could be observed.

The reason for this observation is that the time period is too short to allow morphological changes to occur. In other reports differentiation of mTS cells to the giant cell fate is only clearly observed four days after removal of growth factors (Erlebacher *et al.*, 2004).

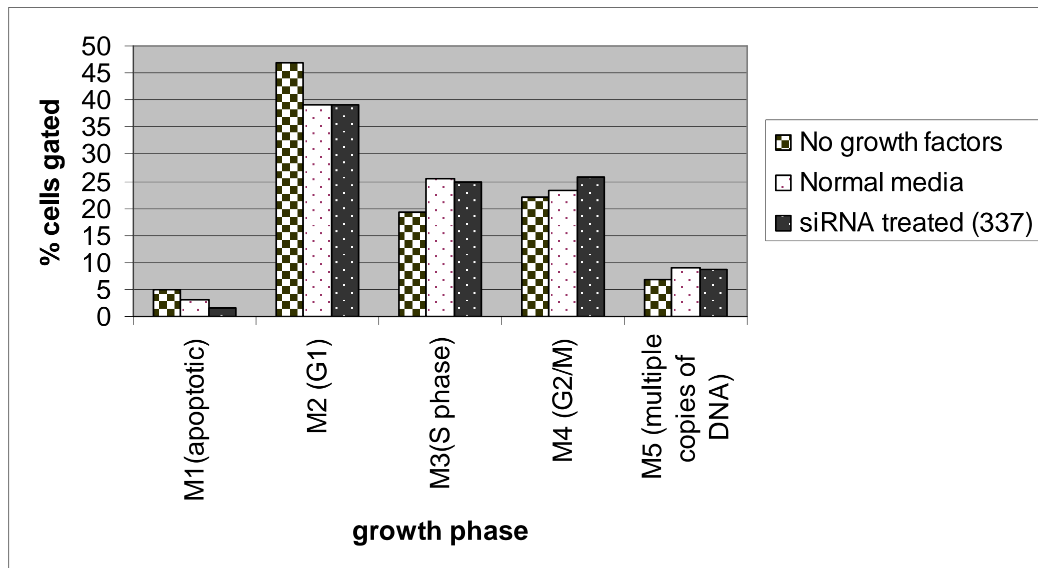


Figure 3.3-3: DNA content analysis by flow cytometry showed no increase in DNA content twenty four hours following Elf5 337 siRNA transfection or growth factor removal. Cells were gated depending on their DNA content and are expressed as a % of the total cells analysed by flow cytometry.

3.4 SUMMARY

Successful siRNA mediated *Elf5* knockdown was achieved. The siRNA which targets nucleotide position 337 in the Elf5 mRNA was optimised to give a greater than 80% knockdown. Furthermore, an interferon response was not induced with siRNA treatment.

Elf5 knockdown and candidate gene expression as a result of *Elf5* reduction were measured using real time PCR. The process from TS cell harvesting, following a siRNA experiment, to real time PCR analysis of the cDNA synthesised from extracted RNA involved many steps (including RNA isolation, DNase treatment and cDNA synthesis). Any one of these can introduce variation into the measured expression of a gene. To mitigate this, a housekeeping gene which is supposed to remain stable over different treatments is also measured and used to normalise samples. A large variation in the expression of the housekeeping gene (β-Actin) suggested there was significant loss of RNA in some of the samples during the steps prior to real time PCR analysis. A second problem observed in this process was the DNA contamination of samples which prevented accurate quantitation of expression. Both of these problems were mitigated following consultation with the manufacturer's guides on the reagents. In

particular changing the protocol to lyse the cells while still in culture dishes with TRIZOL (Invitrogen Life Technologies) considerably improved the quality and consistency of RNA extracted. A recombinant Trypsin enzyme used previously to harvest the cells was probably a source of RNase contamination.

Candidate gene analysis did not result in any genes known to be important for trophectoderm development as significantly changing with *Elf5* knockdown. However *Furin* and *Eomes* could be possible targets as the *p values* calculated from the relative changes in their expression were 0.08 and 0.06 respectively. One factor contributing to the low significance (high *p values*) of candidate genes is the large standard deviation between samples. This is due in part to inherent biological variability, however errors in calculating the relative expression, such as errors in the amplification efficiency are also likely to contribute to this.

The method of calculating the amplification efficiency was not ideal. Four serial dilutions were made of one of the samples being analysed and then the Ct's graphed versus the dilution. Pipetting errors often resulted in graphs with a non-linear relationship. The amplification efficiency estimated from this therefore could contain considerable error. This in turn has a profound error on the calculated relative expression because small errors in the amplification efficiency are amplified in an exponential calculation.

A second source of error is variation in the expression of the housekeeping gene with treatment (Bustin, 2002). A more accurate method of normalisation is the use of a normalising factor calculated from the geometric mean of multiple housekeeping genes (Vandesompele et al., 2002). Validation of the microarray results (Chapter 5) used this method of normalisation.

Morphological changes such as an increase in cell ploidy were not observed. This is most likely due to the short time period (24 hours) following transfection. Changes in morphology (giant cell formation) are not reported to be seen until four days following complete growth factor removal(Simmons et al., 2007).

CHAPTER 4 : ESTABLISHING A SYSTEM FOR INDUCIBLE *ELF5* OVER-EXPRESSION

4.1 OVERVIEW

The principal aim of this study was to determine the down stream targets of the Elf5 transcription factor in mousetrophoblast stem cells (mTs). MTs cells are are *in-vitro* models for early placental development and require Elf5 for their maintenance (Donnison *et al*., 2005). One method of identifying Elf5 target genes is by artificially increasing the level of Elf5 expressed by mTS cells and then searching for changes in candidate gene expression via quantitative real time PCR or by using a microarray to measure global changes in gene expression. The Tet-On system (BD Biosciences-Clontech, USA) was used to try and develop a system, whereby upon addition of tetracycline, Elf5 could be inducibly expressed. Development of a tetracycline inducible expression system for *Elf5* in mTS cells involves three steps:

1. Stable transfection of the inducing plasmid pTet-On and screening for the cell line which produces the greatest induction using a tet-responsive luciferase reporter gene. This was assayed using either the dual or single luciferase assay systems (Promega Corporation, 2005).
2. Construction, using standard molecular biology cloning techniques, of the tetracycline responsive *Elf5* expression plasmid, 'pTRE-Elf5'.
3. Stable transfection of the best pTet-On cell line with pTRE-Elf5 and screening of this using RT-real time PCR to check for best induction of *Elf5* mRNA.

4.1.1 Target Gene Over-Expression Systems

One way of artificially increasing Elf5 expression is to transiently transfect growing cells with a vector expressing the *Elf5* gene or cDNA under the control of a strong mammalian promoter such as the human cytomegalovirus promoter/enhancer (p_{CMV}). However during a transfection experiment not all cells take up the foreign DNA and those that do can take up different numbers of copies; therefore the expression of the exogenous gene varies between cells. To measure gene expression changes using real-time PCR or a microarray cells, are harvested and their mRNA extracted. If all cells from a transfection experiment are harvested, then the effects of the exogenous gene

are averaged across all cells, many of which will not have received any copies of the transgene. One way to obtain a more pure population of transfected cells is to sort the cells using fluorescent activated cell sorting (FACS), however the transfected vector must also code for a fluorescent protein for this system to be used. Long term effects of a change in gene expression cannot be analysed using a transient transfection because as cells grow and divide the number of copies each cell carries is diluted, until eventually the level of exogenous expression per cell is too small to have a significant effect.

These drawbacks can be overcome by stably integrating the gene of interest (*Elf5*) into the genome under the control of an inducible system; such that expression of the gene can be turned on or off. This allows a homogeneous population of cells expressing the *Elf5* gene under the experimenters control to be used in analyzing the effects of increasing Elf5 expression. Because expression is inducible the length of time of 'over-expression' can be controlled, allowing a time course of expression changes to be measured and a suitable control of non-induced cells is easily obtained.

4.1.2 The Tetracycline Inducible system

Various inducible eukaryotic promoters exist can which be used to induce an adjacent target gene's expression in response to a stimuli, for example heavy metal ions, heat shock or hormones. However the effects of the stimuli can also induce undesirable changes in gene expression, induction of the gene be slow (up to days) and can have a low induction due to the leakiness of the commonly used eukaryotic promoters (Gossen *et al.*, 1993). In contrast, use of a tetracycline-response promoter gives tight on/off regulation in a short amount of time (30 minutes), high levels of induction compared to other promoters (Yin *et al.*, 1996) and because it recognises a prokaryotic target sequence it is highly specific and does not induce other genes (BD Biosciences Clontech, 2005)

Tetracycline and its derivatives all share an identical four-ringed carbo-cyclic skeleton. In Prokaryotes tetracycline based antibiotics bind to the 30s ribosomal subunit and prevent protein synthesis. Resistance is obtained in *E.coli* by expressing the TetA protein which exchanges a proton for the outward transport of tetracycline-Mg^{2+} complex from the cytosol. However at high concentrations of TetA protein, cations

are transported at such a high rate the cell can become depolarised and die. Therefore expression of the TetA protein is tightly regulated by the Tetracycline repressor protein (TetR). In the absence of tetracycline the Tet-Repressor protein (TetR) binds to the tet operator sequence and prevents transcription of the tetracycline resistance operon. In the presence of tetracycline the TetR protein cannot bind to the promoter sequence of the tetracycline resistance operon and transcription can proceed (Sambrook & Russel, 2001a).

Gossen and Bujard (1992) fused the TetR protein with the VP16 C-Terminal activation domain of the Herpes Simplex Virus (HSV) to create a hybrid activator protein called the tetracycline-controlled transactivator (tTA). The tTA protein, upon tetracycline removal, could be used to induce expression up to 100,000 fold of a luciferase reporter gene fused to a minimal CMV promoter (lacking the enhancer) and seven repeats of the TetR binding sequence from the tetracycline operator (Gossen & Bujard, 1992). Of interest is the fact that after HeLa cell lines had been established which stably expressed the tTA protein and were screened by transient transfection with the luciferase reporter construct, in those which showed induction of luciferase the tTA protein could not be detected by western blot. This shows that the cells which were active had a very low intracellular concentration of tTA protein. The authors suggest that there could be a selection against effects caused by high concentrations of the VP16-activating domain. One of these positive clones was then stably transfected with the luciferase reporter construct and the cells screened again for their ability to induce luciferase when tetracycline was removed. Once again of interest is the fact that a greater than 500 fold variation in luciferase protein expression (relative light units/μg of protein) was seen both with and without tetracycline induction. The authors suggest this is due to differences in the integration site and the number of units integrated. These observations highlight the need to screen a large number of clones before a suitable clone is found which shows high induction of the response gene and a low background. A disadvantage of the system described by Gossen and Bujard (1992) is that to prevent expression of the response gene, cells must be maintained in a tetracycline containing medium. An advance on this system was later developed (Gossen *et al.*, 1995b) where four amino acids in the tTA protein were exchanged to allow the protein to act in reverse; upon tetracycline addition it bound DNA, and in the absence of tetracycline it was no longer active. This protein is given

the name of reverse tetracycline controlled transactivator (rtTA) and this forms the basis for the tetracycline on (Tet-On) system. More than a 1000-fold induction was achieved using this system.

4.1.2.1 The Tet-On system

The Tet-On system works by firstly the constitutive expression by mammalian cells of the rtTA protein which is stably integrated into the genome. Secondly, a construct containing the gene of interest fused to the tetracycline response element (TRE) which contains seven repeats of the rtTA binding element. Upon addition of tetracycline (or a tetracycline analogue such as doxycycline) the rtTA protein binds to the TRE element and induces expression of the gene of interest. Therefore there are two steps to establishing an Elf5 inducible system in mTS cells: the integration of the rtTA coding sequence into the genome and screening of these to find the best cell line;* followed by stable integration of a construct, where the Elf5 gene is expressed under the control of the TRE element into the best pTetOn cell line selected previously and screening of these clones.

*(Cells can be transfected with a vector carrying the gene of interest and an antibiotic selection marker. Selection over a period of time for cells resistant to the antibiotic gives several cell lines which have integrated the foreign gene into their genome. Integration of foreign DNA into the genome is a random process which is not well understood, however colonies of cells which originated from a single cell precursor can be isolated and analysed for their expression of the exogenous gene.)

4.1.3 Preparation of DNA Vectors Used in the pTet-On System

4.1.3.1 Propagation of Vectors

The pTRE2hyg vector and pTetOn vector supplied by Clontech (BD Biosciences-Clontech, California, USA) were transformed into DH5α competent cells (Invitrogen) following the protocol given in section 2.2.2.4. An isolated colony of each was selected and also grown up overnight in 250ml of LB/ampicillin broth and used to make a maxi-preparation. After the maxi-preparation the purified DNA was resuspended in TE buffer and the concentration measured using the NanoDrop spectrometer (NanoDrop technologies, USA); the pTetOn vector had a concentration of 1500ng/μl and the pTRE2hyg vector had a concentration of 4100ng/μl. To verify the correct

vectors had been transformed the pTetOn and pTRE2Hyg vectors were cut wilt restriction enzyme (section 2.2.2.8) pTetOn was linearised with *Sca*1 to give a characteristic 7.4kb product; pTRE2hyg was cut with *Xcho*1 which cuts twice giving 3.8kb and 1.6kb products. Digests were run on a 1% agarose gel stained with ethidium bromide. The gel shown below after the restriction digest shows the correct vectors has been transformed into the bacteria and isolated successfully (Figure 4.1-1).

The pTRE2Hyg-luciferase plasmid is also supplied in the pTet-on kit (BD Biosciences-Clontech, USA) and expresses a luciferase reporter gene under the control of the tet-response element. This vector can be transiently transfected into cells which are hoped to be expressing the rtTa (tet-activator) protein as a means of screening for induction. The pTRE2Hyg-luciferase plasmid was transformed into DH5α *E.Coli* and then a maxiprep prepared as described in sections 2.2.2.4 and 2.2.2.5. After maxiprep the concentration in TE buffer was 4320ng/μl as measured on the Nanodrop. The pCH110 vector (expressing β-Galactosidase enzyme) and the pRL vector (expressing the *Renillla* luciferase vector) and the pGL3P (positive luciferase control vector) were all obtained in-house as glycerol stocks from J. Bracegirdle. These were streaked on to LB/amp plates and then a colony selected which was used to make DNA maxipreparations. The concentration of pCH110, pRL and pGL3P following maxiprep was 711ng/μl, 1234ng/μl and 490ng/μl respectively. All prepared vector DNA was stored at -20°C.

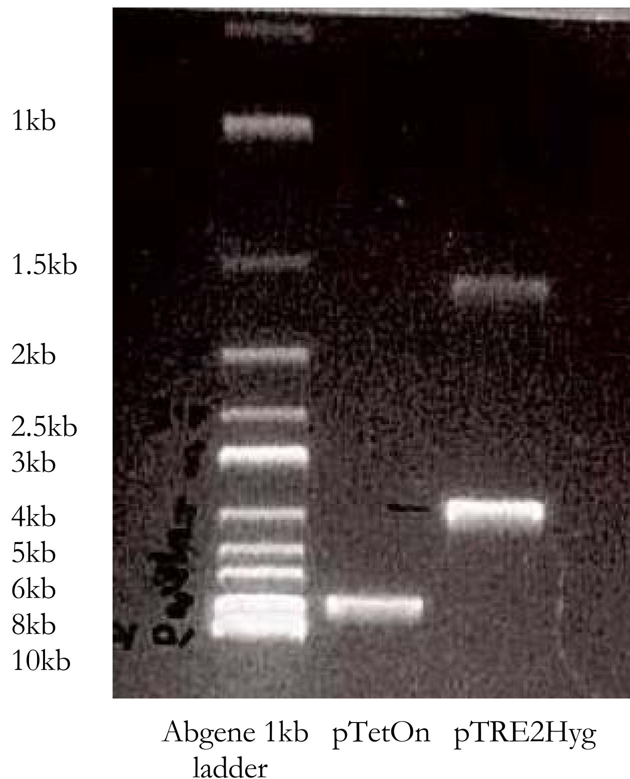


Figure 4.1-1: 1% agarose gel showing pTetOn and pTRE2hyg vectors which had been cut with Sca1 and Xcho1 respectively.

4.1.3.2 Subcloning the Elf5 gene into the pTRE2Hyg Vector

For the inducible over-expression of *Elf5* using the Tet-On system (Clonetech, USA) the *Elf5* cDNA transcript was subcloned into the pTRE2Hyg vector using the methods described in section 2.2.4. The *Elf5* gene was inserted into the pTRE2Hyg vector after the TRE (tet response element) so *Elf5* expression could be controlled by this element.

4.1.3.2.1 Subcloning Strategy

As described in section 2.2.4.1 the vector from which the *Elf5* gene was being removed and the pTRE2hyg vector into which *Elf5* was being inserted, were analysed for compatible restriction sites in the correct orientation. *Elf5* was removed from the 'BRETS-Long' plasmid constructed by P.Huilier/B. Brophy. This vector had been constructed using the pcDNA3 vector (Invitrogen) and an *Eco*R1-*Xho*1 fragment of mouse cDNA containing the Elf5 gene with a 1kb trailer. An excerpt from the BRETS-Long vector is shown below in Figure 4.1-2.

Figure 4.1-2: excerpt from the BRETS-Long vector showing the Elf5 cDNA fragment and flanking restriction enzyme sites (not to scale).

The pTRE2Hyg multiple cloning site (MCS) is shown in Figure 4.1-3. This also contains a *Bam*H1 site at the 5' end; however no matching restriction enzyme site was available at the 3' end. It was decided to cut the *Elf5* fragment with *Xho*1, this gives a 5' overhang which could then be filled in using the Klenow Enzyme (Roche, Germnay) to give a blunt end. The target vector could be cut with *Eco*RV to give a corresponding blunt end.

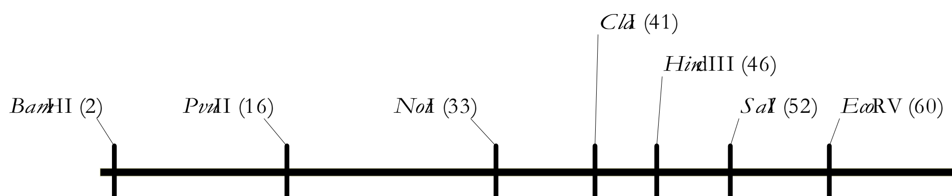


Figure 4.1-3: Excerpt from the pTRE2hyg vector showing the multiple cloning site and restriction sites

4.1.3.2.2 Preparation of the Elf5 cDNA fragment from BRETS-Long

The BRETS-Long vector had previously been transformed into a DH5α strain of E.*Coli*. The glycerol stock of this was streaked on to an LB-ampicillin plate. The following day one colony was selected and used to inoculate 250ml of LB broth containing ampicillin. This was grown up overnight and then used to do a maxi-preparation as described in section 2.2.2.5. The double digest of the BRETS-Long vector was performed sequentially. Firstly BRETS-Long was cut with *Xho*1, and then a Klenow fill in was performed (section 2.2.4.2.2) to produce blunt ends, followed by Geneclean purification (QBiogene, USA; section 2.2.4.2.3). Then a restriction digest using *Bam*H1 was carried out. Restriction digests were carried out according to the

protocol given in section 2.2.2.8. The rationale behind this was so that Klenow (Roche) could be used to fill in only *Xho*1 5' overhang and would not interfere with the *Bam*H1 cut. After this the Elf5 cDNA fragment was separated using a 1% agarose gel and purified from the gel using the Geneclean kit (section 2.2.4.2.5). The agarose gel after the restriction digest is shown in Figure 4.1-4, two products are formed; the 2.2kb Elf5 cDNA segment and the 5.4kb vector.

4.1.3.2.3 Preparation of the pTRE2Hyg target Vector

pTRE2Hyg was cut using BamH1 and EcoRV in a single digestion as described in section 2.2.4.3.1. Two fragments should be produced, one of 5.3kb and the other of only 60bp, as a section from the multiple cloning site is removed. Following the restriction digest the reaction mix was treated with calf intestinal phosphatase (CIP; section 2.2.4.3.2) to prevent the vector self-oligomerizing during ligation. The vector was then purified by running on a 1% agarose gel (Figure 4.1-4) and using the Geneclan kit (QBiogene, USA)

Figure 4.1-4: Digested DNA Fragments for cloning. 1% agarose gel showing cut BRETS-Long (lanes 1 and 2) and cut pTRE2hyg (lanes 3 and 4). The two bands for BRETS-Long correspond to the *Elf5* cDNA fragment (2.2kb) and the 5.4kb vector fragment. Lanes 3 and 4 shown the cut pTRE2Hyg vector, the two DNA fragments are the 5.2kb vector and the 60bp section removed from the MCS, this would have run off the end of the gel due to it's small size.

4.1.3.2.4 Ligation of Elf5 into the pTRE2Hyg vector

T4 DNA Ligase enzyme (Invitrogen) was used to ligate the two DNA fragments together. The protocol is given in section 2.2.4.4. Following gel separation and Geneclean (QBiogene, USA) the *Elf5* DNA fragment was measured using the Nanodrop to have a concentration of 21.9ng/µl and the pTRE2hyg vector had a concentration of 23.2ng/µl. 5.5µl of each DNA fragment was used to give a molar ratio of ~3 moles *Elf5* DNA: 1 mole of pTRE2hyg DNA.

To work out the number of moles used the equation converting µg of DNA to pmol was used:

$$\textit{pmol of double stranded DNA} = \frac{\mu g \textit{ of DNA} \times 1515}{\textit{Number of base pairs}} \qquad \text{Equation 7}$$

0.036pmol of Elf5 DNA (2200bp) was used to 0.037pmol of pTRE2hyg (5200bp). A control ligation was also performed using only 5.5µl of the vector DNA to check for the frequency of self ligation of the vector.

4.1.3.2.5 Transformation and Minipreparation of Ligated DNA

Transformation of both the ligation mix and the control ligation was carried out as given in section 2.2.4.5. The following day the LB/amp plates were inspected to check for the presence of colonies. The ligation control plates had one colony, indicating the CIP reaction had efficiently removed the 5' phosphate residues from the vector. The ligation plates had 5 colonies on the low concentration plate and 30 colonies on the plate spread with a higher concentration of transformed bacteria. 18 colonies were picked and minipreps carried out as detailed in section 2.2.4.6. A clone map of the new vector was created using Vector NTI 10.1.1 software (Invitrogen Corporation 2005) and is shown in Figure 4.1-5 . Restriction analysis of the pTRE-Elf5 vector showed that *Hin*dIII cuts in two positions in the correct vector, giving two fragments; 6.3kb and 1.1kb. If the insert is in the incorrect orientation the fragments will be 6kb and 1.4kb and if there is no insert then only a 5.3kb linear fragment will be produced.

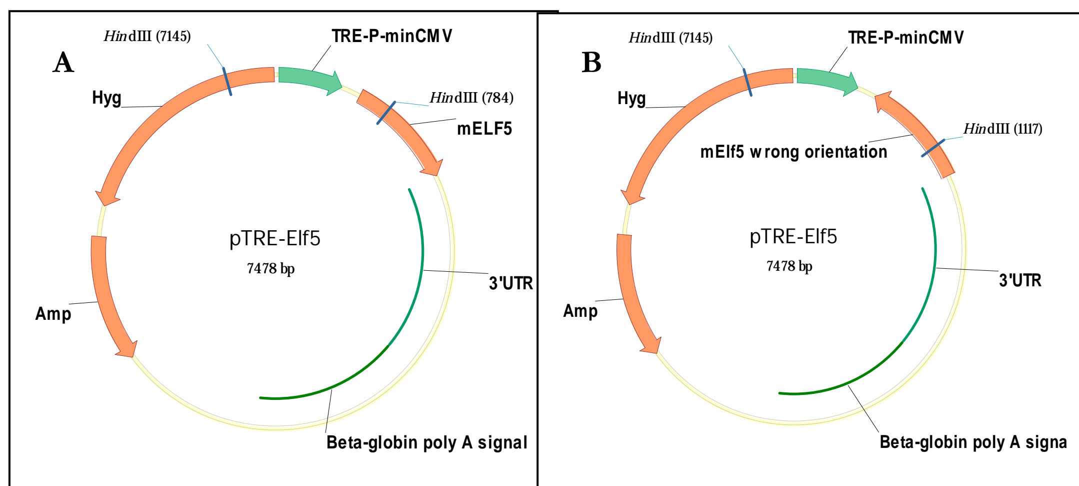


Figure 4.1-5: Clone maps of pTRE-Elf5 showing *Hin*dIII restriction sites (A) when the Elf5 gene has been a correctly inserted and (B) incorrectly inserted.

3µl of miniprep DNA was digested with *Hin*dIII enzyme (section 2.2.2.8). This was then run on a 2% agarose gel alongside a1kb ladder (Abgene, UK). The gel is shown in Figure 4.1-6 . All clones screened contained the insert, giving two bands. All clones also appeared to have the correct sized fragments except clone 1 which it can be seen the smaller of the two fragments was larger than the other clones bands; indicating it was 1.4kb so not in the correct orientation. Clone 10 was selected and grown up for a maxiprep. Some of this was used to make a glycerol stock. The DNA was sent to the Waikato University sequencing facility and sequenced with a forward primer for CMV and a reverse primer for the poly-A globin sequence. The sequencing was successful and verified the sequence was correct.

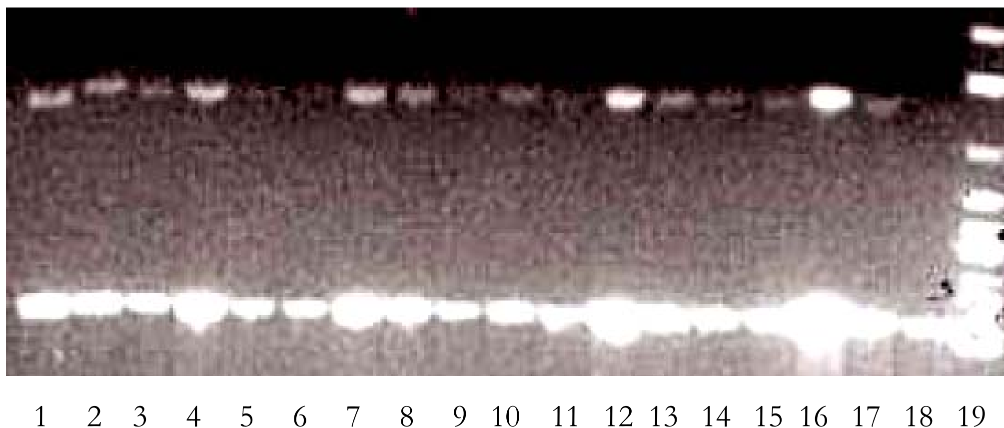


Figure 4.1-6: 1% agarose gel showing 1-18 DNA fragments after digestion of miniprep samples with HindIII, lane 19 is a 1kb ladder (Abgene, UK).

4.2 STABLE TRANSFECTION WITH THE PTET-ON VECTOR

4.2.1 Pilot Experiments

Before the stable transfection was carried out it is recommended in the pTet-On gene expression manual (BD Biosciences Clonetech, available from www.bdbiosciences.com) that the optimal concentration of selection antibiotics and the optimal plating density be determined. The pTet-On vector carries a Neomycin antibiotic selection gene to use for selection in mammalian cells. This gives transfected cells the ability to survive treatment with G418 (Gibco).

4.2.1.1 Optimisation of G418 Concentration

MTS cells were plated at the recommended fixed cell density of 0.34 x10^4cells/cm^2 in 20cm^2 tissue culture dishes. Six different concentrations of G418 were used between 0μg/ml and 800μg/ml as recommended by the pTet-On manual. Cells were grown in 70%CM + F4H + G418 media and the media was replaced every second day. The results are shown in Table 4.2-1: G418 antibiotic selection optimisation for mTS cells.

Table 4.2-1: G418 antibiotic selection optimisation for mTS cells

Concentration of G418 (μg/ml)	Day 1 (% dead compared to 0μg/ml)	Day 2 (% dead compared to 0μg/ml)	Day 3 (% dead compared to 0μg/ml)	Day 4 (% dead compared to 0μg/ml)	Day 5 (% dead compared to 0μg/ml)
0	0	0	0	0	0
50	0	0	0	0	0
100	0	0	0	0	0
200	0	0	0	0	0
400	0	0	0	0	0
800	0	0	0	<5	<5

After day 5 it was observed there was still very little cell death between the cells treated with 0μg/ml G418 and those treated with the highest concentration of G418. The cells growing at 0μg/ml and 50μg/ml were also nearing confluency. All cells were then subcultured and split 1:20 into new 20cm^2 dishes. The experiment was continued for two more days before it was noted that the 0μg/ml and 50μg/ml plates were again reaching confluency. It was decided to abandon the experiment as the range of G418 concentrations although slowing growth at the highest concentrations was not causing cell death. The pTet-On manual suggests that 'massive cell death' should occur at around day 5; however this was obviously not occurring in the range trialled. A new experiment was begun using six concentrations of G418 between 0μg/ml and 1500μg/ml. MTS cells were plated into 4cm^2 wells at a fixed cell density of 3x10^3cells/cm^2. The results from this experiment are shown in Table 4.2-2.

Table 4.2-2: G418 antibiotic selection optimisation for mTS cells, second experiment

Concentration of G418 (μg/ml)	Day 2 (% dead compared to 0μg/ml)	Day 4(% dead compared to 0μg/ml)	Day 6 (% dead compared to 0μg/ml)	Day 8 (% dead compared to 0μg/ml)
0	0	0	0	0
200	0	0	0	0
400	0	0	0	0
800	<5	<5	<5	5
1000	5	5	10	20
1500	10	10	20	30

At day nine the cells were confluent in the lowest three concentrations of antibiotics used. Massive cell death was still not observed at any of the antibiotic concentrations. It was decided to contact James Cross (University of Calgary, Canada) from whom the mTS cells had been sourced to ask if he had any experience using G418 on mTS cells. Doctor Cross informed us that the mTS cells were derived from the ROSA-26 strain mice. These mice constitutively express a neomycin-βGalactose fusion protein and are therefore G418 resistant. An alternative strategy was then decided upon: to co-transfect the pTet-On vector with a puromycin antibiotic resistance vector. If a high molar ratio of pTet-On vector to puromycin resitance vector is used, for example 10:1, then there is a high chance that cells resistant to puromycin will also be carrying the pTet-On vector.

4.2.1.2 Optimisation of Puromycin Antibiotic Selection Concentration

MTS cells were plated out at a fixed concentration of $3.3x10^3$cells/cm^2 in six $4cm^2$ wells. 70%CM + F4H media was added containing different concentrations of puromycin in the range 0μg/ml to 10μg/ml as recommended by the Tet On gene expression systems manual. The results are shown below in

Table 4.2-3.

Table 4.2-3 Optimisation of puromycin antibiotic for selection in mTS cells

Concentration of puromycin (μg/ml)	Day 1 (% dead compared to 0μg/ml)	Day 2(% dead compared to 0μg/ml)
0	0	0
1	80	100
2.5	100	100
5	100	100
7.5	100	100
10	100	100

The minimum concentration which caused cell death was 1μg/ml of puromycin. I was surprised at the rapid cell death, and I thought perhaps this was due to the medium containing puromycin before the cells had even adhered to the plate. The experiment was repeated, however the cells were allowed to adhere for a day in media free of antibiotics before puromycin was added the following day. The same results were observed. A literature search also gave the optimal concentration of puromycin for selection of mTS cells as 1μg/ml (Quinn et al., 2006).

4.2.1.3 Optimisation of Plating mTS Cell Density When Using Puromycin Selection

If cells are plated at too high a density during antibiotic selection, cells will reach confluency before selection takes effect (BD Biosciences Clontech, 2005). Optimal plating density depends on cell doubling time and cell surface area. Following the recommendations in the pTet-On manual mTS cells were plated out at cell densities varying from $3.3x10^5$cells/$4cm^2$ well to $0.063x10^5$cells/$4cm^2$ well. The results are shown in

Table 4.2-4.

Table 4.2-4: Opimisation of plating density when using puromycin selection in mTS cells

Plating Cell Density at Day 0 in a $4cm^2$ well ($x10^5$ cells)	Day 1	Day 2	Day 3	Day 4
3.3	confluent	confluent	confluent	confluent
0.6	Small amount of death	60% dead	90% dead	All dead
0.3	Small amount of death	90% dead	All dead	All dead
0.13	Small amount of death	90% dead	All dead	All dead
0.06	All dead	All dead	All dead	All dead

The highest plating density which still caused massive cell death before the cells reach confluence was $0.6x10^5$cells/$4cm^2$. This corresponds to a cell plating density of $9x10^5$cells/$60cm^2$ dish.

4.2.1.4 Conclusions from pilot experiments

The mTS cells were found to be resistant to G418 selection; high concentrations of G418 were used in an attempt to cause cell death and after communication with James Cross it was discovered that the cells express a neomycin resistance fusion protein which explains the results. As an alternative it was decided to use puromycin antibiotic selection, by cotransfecting with pTet-On and a puromycin resistance cassette. The optimum concentration of puromycin to be used for antibiotic selection was found to be 1μg/ml. This value was confirmed in the literature (Quinn et al., 2006). The optimal

plating density was found to be $9x10^5$cells/60cm^2 dish. The stable transfection will be carried out in a 10cm^2 dish which had previously been plated at a density of $4x10^5$cells. MTS cell have a doubling time of approximately 24hours (Erlebacher et al., 2004). By the time these cells are ready to be plated out for selection this will be two days after initial seeding; each well will contain approximately $16x10^5$cells. Cells can then be subcultured from each well into two 60cm^2 dishes to give a seeding density of $8x10^5$cells/60cm^2, which is slightly below the optimal density. This means cells will be diluted 1:12, this is in agreement with the Lipofectamine 2000 protocol (Invitrogen Life Technologies, , 2006) which suggests a dilution of 1:10 or greater during establishment of a stable cell line.

4.2.2 Transfection and Screening of mTS cells to create a stable line expressing the pTet-On vector

The first step in establishing an inducible cell line is the establishment of a cell line expressing the rtTa protein. A total of three separate co-transfection experiments were carried out on the mTS cells with the pTet-On vector (which contains the rtTa coding sequence) and the puromycin resistance cassette. Transfections were carried out as described in section 2.2.1.7 for establishing a stable cell line; briefly transfections were carried out in 6-well plates with one well non-transfected to serve as a control. The following day each well was subcultured to give a low concentration of cells to allow for selection. Twenty four hours later puromycin was applied at 1μg/ml. Although the mechanism is not well understood the foreign DNA can be randomly inserted into the genome of a transfected cell. A molar ratio was used so there would be a higher number of pTet-On vectors in relation to the puromycin cassette to increase the chance that cells resistant to puromycin would also carry the rtTA gene. The puromycin resistant cell colonies were then screened with a luciferase reporter gene under the control of the TRE element to find the cell line with the greatest ability to induce expression in the presence of doxycycline. The specific details relating to each transfection experiment and their results are given below.

4.2.2.1 The first pTet-On co-transfection

Mouse TS cells obtained from James Cross and at passage 2 at Ruakura were plated into one six well plate at the optimal density of 4 $x10^5$ cells per well. The following day cells were transfected using a molar ratio of 10 pTet-On : 1 puromycin. The pTet-On

vector is 7.4 kb. The Puromycin resistance construct was supplied by Craig Smith and I was informed it was 6kb.

There are two ways of selecting a clone of cells which are resistant to the selection antibiotic. One method is to dilute the cells and plate them into a 96 well tissue culture dish at a concentration which will give 1-2 cells per-well. The other method is to plate cells at a low density into a single large plate. Both methods aim to plate cells at a very low density so that any resistant colonies which form have a higher chance of being derived from a single cell. As this was the first time a stable transfection had been carried out both methods were tried. The day after transfection cells were treated with TRYple (Invitrogen) and a cell count performed. Each transfected well of cells was then diluted to give a concentration of 200 cells/ml. 10µl of the diluted cell suspension was pipetted into each well of a 96 well tissue culture plate and then 150µl of 70% CM + F4H added. This should dilute cells so that 1-2 single cells should be present in each well. One 96 well dish was used per transfected well. One 100mm dish per transfected well was also plated with approximately 1000 cells per dish. The following day the media was changed and 1µg/ml of puromycin added. The media was changed every second day always with puromycin. 96 well plates were inspected for colonies; wells containing greater than one colony were not used. 10 days after first adding antibiotics colonies of cells were clearly visible and were selected. 12 large colonies were 'picked' as described in section 2.2.1.7.5 from the 100mm dishes and the wells containing a single colony in the 96 well dishes were subcultured. All new cell lines were plated individually into a well of a 6-well plate. After 8 days these had grown sufficiently to be frozen down until they could be screened with the luciferase reporter assay.

4.2.2.2 The Second pTet-On Transfection

The procedure was exactly the same as for the first transfection, except mTS cells were at passage 6 at transfection and selection was only carried out by plating each well into two 100mm plates; this was because this method of selecting an antibiotic resistant cell line was more successful in the first co-transfection experiment. It was observed that a low number of cells plated as single cells in the 96 well plate format went on to grow and divide, and more clones could be obtained by plating cells post-transfection in 100mm plates. After the second transfection the individual cell lines were grown up in 6-well plates and frozen down. They were later removed and screened for inducibility using the luciferase reporter assay. Cell lines were labelled by transfection number

followed by line number, for example cell line 1.1 refers to a cell line from transfection 1, cell line 1.

4.2.2.3 Screening of Cells from Transfections One and Two

Prospective pTet-On mTS cells were screened using a co-transfection of pTRE2Hyg-luc (firefly luciferase response vector) with the pCH110 vector (expresses β-Galactosidase constitutively). A 1:1 mass ratio was used between pTRE2Hyg-luc and pCH110, which corresponded to a 1:1 molar ratio since the vectors are about the same size (7kb and 7.1kb respectively). Each cell line was grown up and used to seed two wells of a 12-well plate. They were then transfected as described in section 2.2.1.7.6 except that for the first assay none of the controls were included. Following transfection one of the wells for each line had doxycycline added (1μg/ml) and the other was the control well to compare for induction. 48 hours after transfection of the reporter gene constructs cell lysates were harvested and assayed for luciferase expression (section 2.2.2.1) and β-Galactosidase expression (section 2.2.2.2). Luciferase measurements were made in triplicate and β-Galactosidase measurements made in duplicate. For each cell line luciferase expression was normalised by dividing it by β-Galactosidase expression. The normalised induced luciferase expression was then divided by the non-induced luciferase expression level to give the relative induction for each cell line. Cells were screened in batches of six to ten and in total 48 cell lines were tested for luciferase induction from stable transfections one and two.

Early after the first screening experiment it was discovered that the mTS cells were derived from a mouse line which constitutively expresses β-Galactosidase (the Rosa26 mouse line; James Cross, personal communication). Therefore β-Galactosidase is not ideal as a normalising reporter gene, as it is more correlated to absolute cell number than transfection efficiency. This will especially be the case if the transfection efficiency is low. However at high transfection efficiency the transient expression of β-Galactosidase would be a more dominant component of the total β-Galactosidase expression. Due to the expected induction being greater than a hundred fold, in a good cell line, it was thought that β-Galactosidase normalisation although not ideal, would suffice.

4.2.2.3.1 Inducible Luciferase Results

Raw luciferase results from the first 13 cell lines obtained from the first transfection are shown below in Figure 4.2-61. The maximum normalised induction was around 3 fold in line 1.5. Upon inspecting the raw measurements it was found that cells which expressed higher levels of induced luciferase also had high basal levels of luciferase (without doxycycline). It was thought that perhaps the high background levels could be masking the true induction.

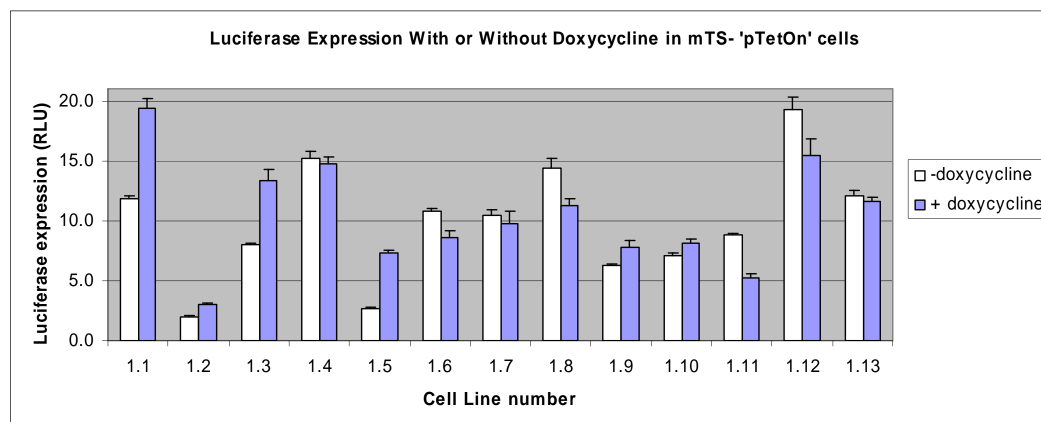


Figure 4.2-1 Induction of a luciferase reporter gene under the TRE control in the antibiotic resistant cell lines. The average luciferase expression as measured by the luminometer is shown for each cell line (1.1-1.13) +/- sem, n=3.

4.2.2.3.2 Trouble Shooting 1:

Tetracycline contamination of Foetal Calf serum (FCS) used in the media can be a problem (BD Biosciences Clontech, 2005) causing expression of the activation of the response gene without addition of tetracycline/doxycycline. A small amount of certified tetracycline free FCS was supplied in the pTet-On kit, so this was used to make media and then a few cell lines which appeared to show some induction from the first experiment were tested for induction. The graph below shows that the normalised induction was not increased when the certified Tetracycline free medium was used. From this it was concluded that tetracycline contamination in the medium was not a problem.

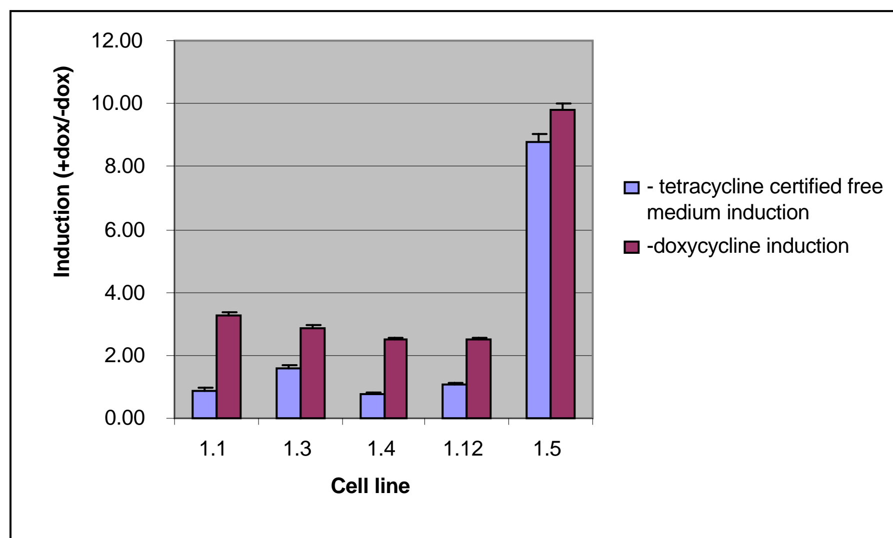


Figure 4.2-2: luciferase induction in cell lines 1.1, 1.3, 1.4 , 1.12 and 1.5. Cell lines were grown in certified free tetracycline medium to see if there was any increase in induction when compared to induction in normal medium without doxycycline added. Luciferase expression was normalised to β-Galactosidase. Shown is mean induction level +/- sem, n=3.

4.2.2.3.3 Trouble Shooting 2

The next approach to ascertain the reason for the low level of induction was to test the doxycyline stock was sufficient to give maximum induction at the concentration used (1μg/ml). Doxycyline was made up in water to a concentration of 1mg/ml and, stored at -20°C, can last up to 1 year. However it could have gone off and therefore be limiting induction. To test the doxycycline, different concentrations of doxycyline were used to induce expression of luciferase in mTS cells co-transfected with pTet-On and pTRE2hyg-Luc (as well as β-Galactosidase for normalisation). Figure 4.2-3 shows luciferase expression in response to different concentrations of doxycyline inducer. From 1μg/ml to 2μg/ml of doxycycline there was no increase in luciferase expression indicating doxycyline at 1μg/ml was not limiting induction.

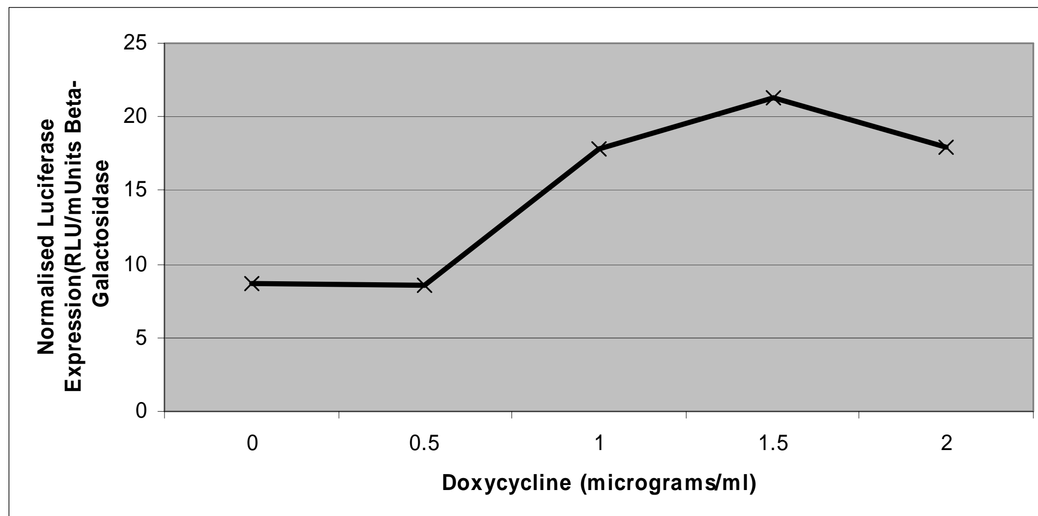


Figure 4.2-3: Normalised luciferase reporter expression in the presence of varying concentrations of doxycycline inducer

4.2.2.3.4 Trouble Shooting 3

As the concentration of doxycycline in 'no doxycycline medium' did not increase the background level and the concentration of doxycycline used to induce luciferase expression was not limiting, the next step was to include a range of controls to try and ascertain the levels of luciferase which should be expressed (using a positive luciferase control) and to see if the background levels of luciferase was inherent, due to auto-luminescence from the cells, or due to leakiness of the TRE-Luciferase vector. The controls tested were:

1. To test that the luciferase assay was working and as an indication of the possible expression levels of luciferase a CMV-luciferase positive control plasmid (pGL3P) was obtained from J. Bracegirdle. This vector expresses the luciferase gene under the expression of a CMV promoter, however it lacks an enhancer. It therefore will express luciferase, however only basal amounts. If the luciferase expression from 'PTet-On' cells is not higher than this then it would appear induction is not occurring in the cells.

2. A transient co-transfection with both the pTet-On vector and the pTRE-2hyg-luc response vector in the presence of doxycycline to give an idea of the maximum induction achievable; as neither rtTA protein or luciferase response vector will be limiting in this scenario.

3. Transfection with only pTRE-2hyg-Luciferase to give an idea of the basal expression of luciferase without the presence of any rtTA protein and therefore to test the leakiness of the pTRE-Luciferase vector.

Figure 4.2-4 shows the luciferase expression of some control samples. Mouse TS cells which had not previously been transfected with the pTetOn vector were co-transfected with the pTRE-Luciferase response vector and the pTetOn vector to get an idea of the maximum induction in the presence of doxycyline. As can be seen, the maximum induction (with doxycycline/no doxycycline) is around four fold. The TRE-Luciferase expression is about the same as the non-induced sample. The Luciferase CMV positive control vector had a higher expression than the positive induction sample. The negative control was not transfected with either plasmid and had an auto-luminescence below the detectable level of the luminometer.

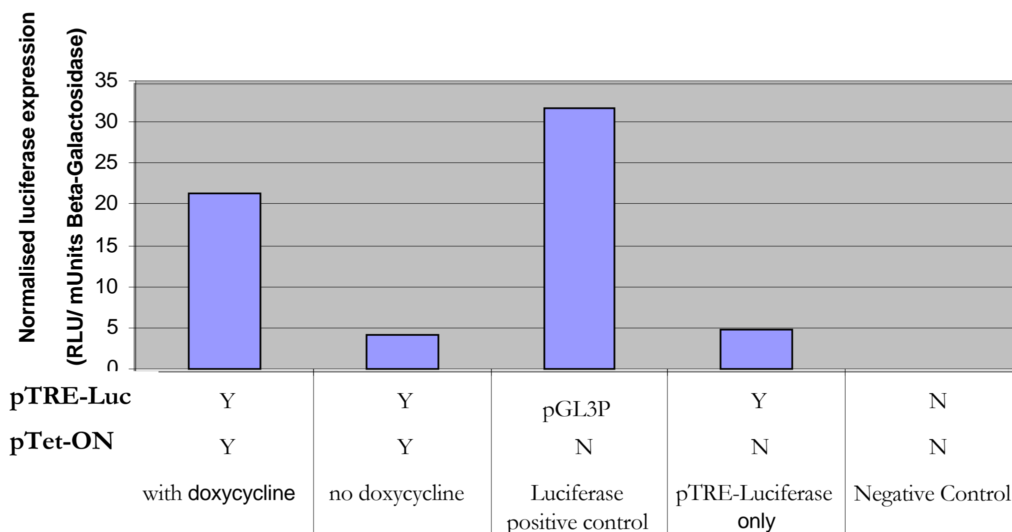


Figure 4.2-4: Normalised Luciferase values for controls. The combination of vectors used in each transfection is shown. All transfections were done in mTS cells which had not been previously transfected with the pTet-On vector and a β-Galactosidase vector was included for normalisation. ?; expected to contain pTetOn on the basis of puromycin resistance.

4.2.2.4 Dual Luciferase Assays

As discussed earlier luciferase results were normalised to β-Galactosidase expression, however β-Galactosidase was already expressed by the cells making it a less than ideal reporter gene. As the induction levels were not as high as expected a second method of

normalising the firefly luciferase was sought. The dual luciferase system (Promega, USA) uses the fact that the Firefly luciferase enzyme and the *Renilla* luciferase enzyme have distinct substrates. The firefly luciferase enzyme catalyses the oxidation of beetle luciferin using ATP while the *Renilla* luciferase enzyme catalyses the oxidation of coelenterate-luciferin (Promega Corporation, 2006). This means both luciferase enzymes can be assayed in one reaction mix in the luminometer; first the firefly luciferase luminescence is measured, this reaction is quenched and then the *Renilla* luciferase is measured. Both assays are very sensitive and linear over seven orders of magnitude. The normalising vector used was pRL-CMV, which expresses *Renilla* luciferase under the control of a CMV promoter/enhancer. The dual luciferase assay was carried out as described in section 2.2.2.3. During the assay both the luminescence due to the inducible firefly reporter vector and the *Renilla* luciferase vector were measured. The expression of the firefly luciferase enzyme (RLU) was then divided by the Renilla expression (RLU) to give a normalised ratio used in inducible calculations.

4.2.2.4.1 Dual Luciferase Results

The first time the dual luciferase assay was performed on cell lysate samples it was found the expression of Renilla far exceeded that of the firefly expression. The transfection was carried out using a 1:1 mass ratio of pTRE2Hyg-luc to pRl-CMV for each cell line as described in section 2.2.1.7.6. However the expression of *Renilla* was up to 10 fold greater than the firefly luciferase expression (Results not shown). The dual luciferase manual (Promega Corporation, 2006) states that "*trans* effects between promoters on co-transfected plasmids can potentially affect reporter gene expression" and this is especially the case when either/both vectors contain strong promoter and enhancer elements. Promega recommends using a relatively small quantity of the control reporter vector. As both vectors contain strong viral promoters the next experiment carried out used a 5:1 ratio of TRE2Hyg-luc to pRl-CMV.

The results from the first assay are shown in Figure 4.2-5. The two cell lines which had previously been identified as possibly carrying the rtTA gene were screened alongside the three controls described in section 4.2.2.3. The induction from cell lines 1.5 and 2.37 was 1.3 fold and 1.2 fold respectively. The expression of the *Renilla* vector however was still around 5 times the expression of the firefly luciferase. The inducible control (pTetOn and pTRE2Hyg-luc transiently transfected) showed a maximum induction of about 10 fold. Of interest was the fact that the leakiness control (simply

transient transfection with pTRE2Hyg-Luc) had a similar level of un-induced expression as the inducible control in the absence of doxycycline.

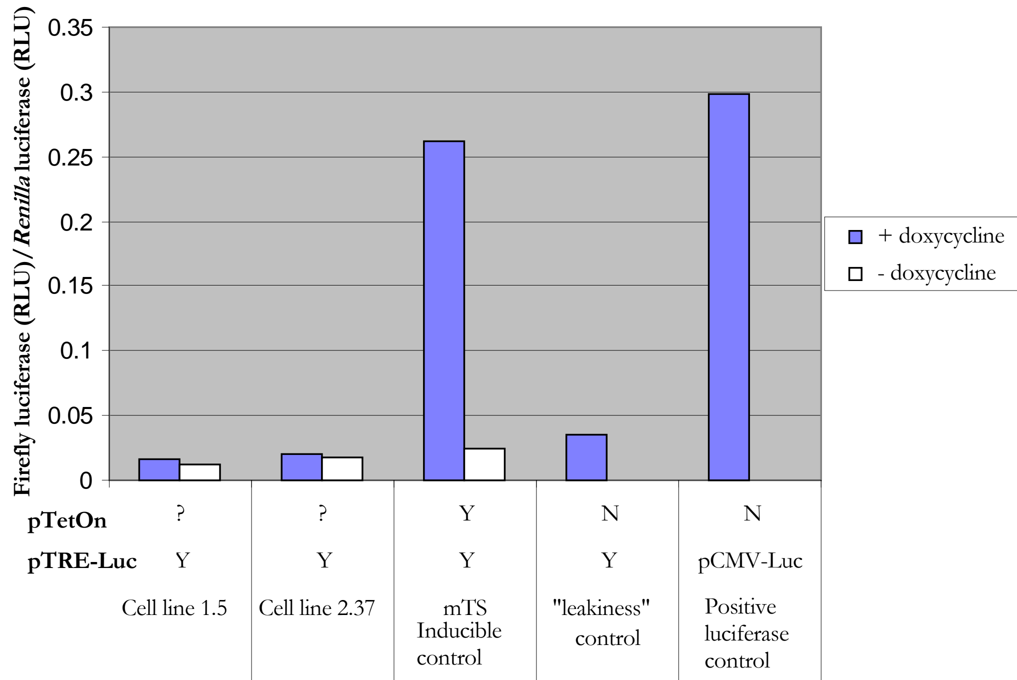


Figure 4.2-5:Results from the dual luciferase assay. Firefly luciferase expression normalised to renilla luciferase expression is shown in the presence or absence of 1μg/ml doxycycline for cell lines 1.5 and 2.37. The results from the controls checked in the assay are also shown. The controls were all mTS cells which had previously not been transfected with the pTetOn vector. The combination of plasmids transiently transfected into the controls is shown.

All isolated cell lines were screened using the dual luciferase assay system except a 10:1 ratio of pTRE2Hyg-luc was used to pRl-CMV. The ratio of normalised firefly luciferase in the presence or absence of doxycyxline for all cell lines screened is shown in Table 4.2-5. The results show that none of the cell lines screened show an acceptable level of induction. It was decided to perform a third stable transfection.

Table 4.2-5: Dual Luciferase results. Induction of 'pTET-On' cell lines determined by induction of firefly luciferase normalised to Renilla luciferase expression. Cell lines previously identified as possibly displaying the inducible phenotype have been shaded.

Cell line	Normalised firefly luciferaseInduction (+dox/-dox)	Cell line	Normalised firefly luciferaseInduction (+dox/-dox)
1.2	1.18	2.107	0.91
2.44	0.83	2.37	1.46

2.98	0.79	1.3	1.09
2.86	1.63	2.26	0.89
2.17	0.34	2.32	1.70
2.21	1.57	2.74	1.28
2.42	0.78	2.40	0.77
1.1	0.72	2.22	0.78
2.2	2.14	2.72	0.94
2.75	1.10	2.23	0.45
1.13	0.46	2.36	1.06
2.93	0.52	2.24	0.98
2.21	0.76	2.46	0.92
2.77	0.57	2.30	0.71
2.73	1.02	1.8	0.77
2.31	1.09	2.92	1.80
1.3	1.22	2.103	1.11
1.20	0.70	2.25	1.05
1.14	0.36	2.99	1.35
1.7	1.12	2.35	1.65
2.93	0.63	2.41	0.37
1.4	1.11	1.5	1.32

4.2.2.5 pTetOn stable Transfection Three

The previous transfections had been carried out using a circular form of the pTetOn vector. It was suggested that a linear form of the vector could be more successful. The pTetOn vector was digested using the *Sca*I restriction enzyme which cuts in a single location using the protocol described in section 2.2.2.7. The cut vector was purified using a phenol/chloroform extraction and ethanol precipitation. The resulting purified plasmid was resuspended in 20µl of TE buffer. The concentration as measured by the Nanodrop was 1.5µg/µl. After cutting the linearised pTetOn vector was run on a 1% agarose gel to check for complete digestion. The linearised puromycin cassette DNA supplied by C. Smith to be used for the co- transfection was also run along with its undigested precursor. The gel is shown in Figure 4.2-6. As it can be seen from the gel, the pTetOn was cut and of the correct size (7.4kb). The uncut vector had a fuzzy band due to super-coiling. The cut puromycin vector was 1.4kb, and the uncut version was 4.7kb. This was a surprise as it had previously been though that the cut puromycin vector was simply linearised, however it had been digested to remove only the 1.4kb selection cassette.

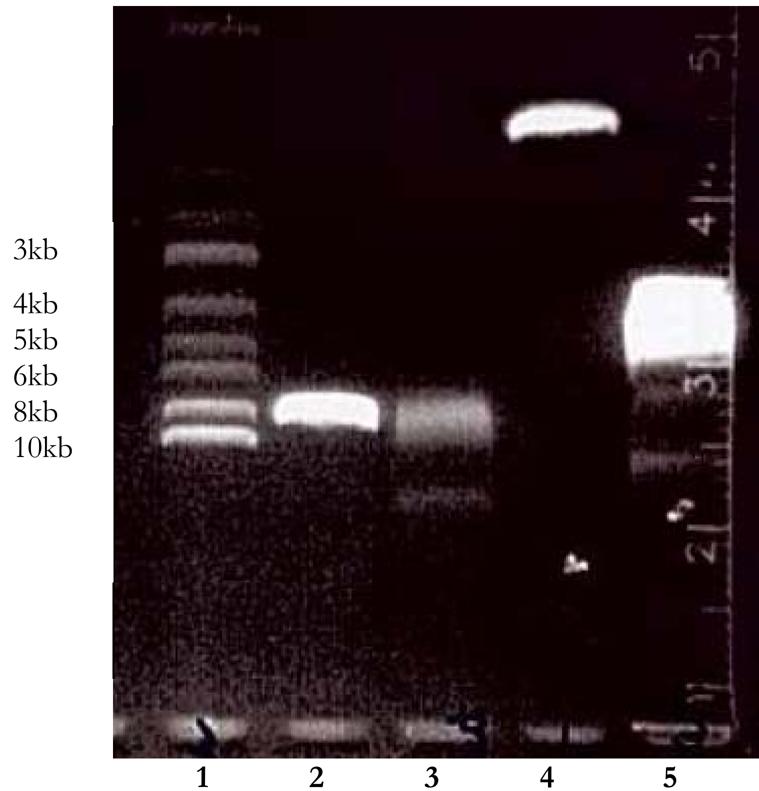


Figure 4.2-6 1% agarose gel showing efficient restriction enzyme digest of the pTetOn and puromycin vectors.1) Abgene (UK) 1kb DNA ladder, 2) pTetOn linearised with *Sca*I digest (7.4kb), 3) pTetOn uncut, 4) puromycin vector cut (1.4kb), 5) puromycin vector uncut (4.7kb).

The stable transfection was repeated at a molar ratio of 4 pTetOn vector to 1 puromycin cassette vector; transfection conditions and growth/selection of stable cell lines was carried out as described in section 2.2.1.7 and section 2.2.1.8. The ratio used of pTetOn vector to puromycin selection vector was lower than intended because the volumes of DNA used had been calculated using a size of 6kb for the puromycin. Because of this and the low success of obtaining an inducible cell line from previous stable transfections it was decided to first screen the cell lines using PCR to check if they were carrying the rtTA gene. During expansion of the cell lines, each cell line was plated into two wells of a six-well plate. When these wells were approximately 70-90% confluent one well was used to freeze down the cell line and the other was treated with TRYple (Invitrogen) and then screened for presence of the rtTA gene as described in section 2.2.1.9. In total 115 cell lines were isolated.

4.2.2.5.1 PCR Screening for the rtTA gene: Results

Following crude extraction of genomic DNA from each cell line, five samples were selected and 1µl of each analysed on the Nanodrop (Nanodrop technologies, USA) for concentration, as about 100ng of DNA is best for a PCR reaction. The samples contained a wide range of DNA but the average was about 300ng/µl (data not shown). Thus 0.5µl was used as template each PCR reaction. Following the PCR, 8µl of each reaction was run on a 2% agarose gel. As a positive control 1.4ng of pTet-On plasmid was used for PCR and electrophoresed. The negative control was genomic DNA extracted alongside the sample from several wells of mTS cells and mEFs which had not been transfected with the pTetOn vector. An example of one of the gels is shown below.

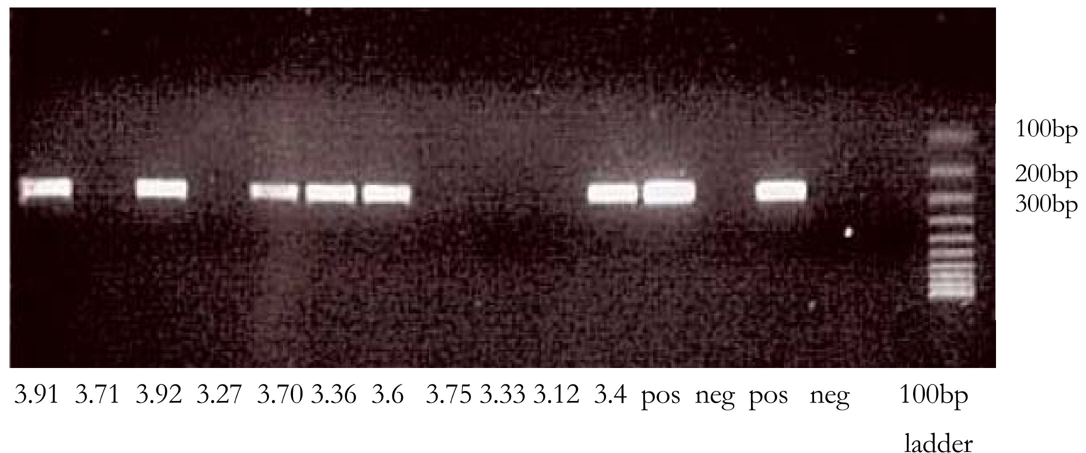


Figure 4.2-7: PCR Screening for rtTA positive controls. A 2% agarose gel loaded with the PCR product using rtTA primers with genomic DNA from several puromycin resistant cell lines (cell lines labelled 3.91-3.4). A positive control (pTetOn plasmid DNA) and negative control (non-transfected cell lines) were also run. The last lane is a 100bp ladder (Abgene) the rtTA pCR product should be 260bp.

In total 58 rtTA positive clones were identified and 56 negative clones. The negative clones were discarded. The low level (50%) of positive clones detected was most likely due to the higher amount of puromycin selection cassette used than intended. Due to time constraints the positive cell lines could not be screened for their ability to induce the pTRE2Hyg-Luc expression.

4.2.3 Discussion on Establishment of a mTS *Elf5* inducible Cell Line

A cell line inducibly expressing Elf5 using the pTetOn system was unable to be established due to difficulties in establishing a cell line which expressed the rtTA protein in a form which could inducibly activate a firefly luciferase reporter gene. However the *Elf5* gene was successfully cloned into the pTRE2Hyg vector to create a vector which will be able to express *Elf5* when bound to by the rtTA.

Problems with the doxycycline inducer were ruled out as a reason why no induction was seen in mTS cell lines previously co-transfected with the pTetOn vector which carries the rtTA protein sequence. Induction in mTS cells transiently cotransfected with the pTetOn vector and a TRE-luciferase reporter plasmid reached a maximum at 1μg/μl doxycycline (Figure 4.2-3). This is also reported in the literature (Gossen et al., 1995b) where induction using rtTA began at concentrations above 100ng/μl doxycycline, and was maximum at 1μg/μl or above.

It is believed the primary reason for not being able to establish a suitable mTS-pTetOn cell was the unwitting use of a high ratio of the puromycin selection cassette relative to the co-transfected pTetOn vector. The puromycin cassette was originally thought to be 6kb in length, however after running the transfected vector on an agarose gel it was found it was only 1.4kb, as the resistance gene had been cut out of the vector. This caused a high proportion of cells to carry only the antibiotic selection marker and not the pTetOn DNA. For the third transfection a ratio of 5:1 was used of pTetOn : puromycin, 50% of the antibiotic resistant cell lines isolated from this transfection carried the rtTA gene (coded by the pTetOn vector). For the first two transfections the true ratio used of pTetOn to puromycin was actually 2.5:1. This correlates to 25% of cell lines isolated from transfection one and two could have carried the rtTA gene. In total 48 cell lines were screened from stable transfections one and two, meaning about 12 of these lines could have carried the rtTA gene.

Apart from the primary reason of an incorrect transfection ratio, there are several possible reasons why none of the cell lines isolated exhibited the inducible phenotype to a satisfactory level. The first is that some cell types may not be suitable for establishing the pTetOn system (Ackland-Berglund & Leib, 1995). Also it is recommended that at least 30 cell lines need to be screened before a suitable line is

found (BD Biosciences Clontech, 2005). In a similar experiment, co-transfections with the pTetOn vector and a G418 resistance cassette in HeLa cells gave an inducible phenotype in 10% of cells screened using a TRE-luciferase gene (Gossen *et al.*, 1995a). If the same rate of success was achieved then only 3-4 mTS-pTetOn lines should show the inducible phenotype. In all HeLa cell lines which did have an inducible phenotype the tTA protein was not detectable by Western blots, showing it is expressed at a low level (Gossen & Bujard, 1992). The authors believe the reason for this is that high expression of the rtTA protein can be toxic, due to its VP16 activating domain. High numbers of cell lines need to be screened to find cell lines which are capable of inducing expression in the presence of tetracycline yet have copies of the rtTA gene integrated in chromosomal locations such as to be expressed at non-toxic levels.

Finally, it has been observed that in transient transfections used to screen for rtTA active cell lines the level of induction is never as high as in cell lines which have the tet responsive gene stably integrated into the genome. This is because the background level of expression of the TRE-reporter gene is high in transient transfections where a high amount of DNA is used. A high level of TRE-reporter gene DNA can also titrate out the activator protein (Gossen et al., 1995a). In a stable system the TRE-gene is able to be integrated into a 'transcriptionally silent but activatable' region, giving low levels of background and the high levels of induction sought (>1000 fold, (Gossen et al., 1995b)). For example it has been reported that transfection of HtTA cells in a 10cm^2 dish with 2µg of DNA gave only a 155-fold induction, however with 1ng the induction was 1000-fold (Gossen et al., 1995a). In the screening experiments performed here the amount of DNA used was that recommended by the lipofectamine protocol (Invitrogen life technologies, 2006), which suggests 1.6µg for a 4cm^2 dish. It was also noticed that the level of background expression in un-induced samples of the inducible control was about that of only the pTRE2Hyg-luc gene (Figure 4.2-4 and Figure 4.2-5). This suggests that the background expression of luciferase was due to the pTRE2Hyg-luc leakiness and not due to the rtTA gene activating luciferase expression in the absence of doxycycline. From the literature this leakiness is most likely due to the high amounts of DNA transfected.

It was interesting to note that although using the first luciferase assay some cell lines did look like they could be working in an inducible fashion these same cell lines when screened later in the dual luciferase assay showed very low levels of induction. This

could be due to the problem of using β-Galactosidase as the normalising gene, when it was constitutively expressed by the cells. However it is also known that expression of a transgene is often silenced when the cell line is cultured over some time (Pagliaro & Praestegaard, 2001). The cell lines which showed some promise of being inducibly active were screened several times using the first luciferase assay before they were later grown up and screened again with the dual luciferase assay. This means some cell lines were perhaps 10 passages older when screened using the dual luciferase assay. Another point is the *Renilla* expression plasmid used as a 'normaliser' was driven by the strong CMV promoter/enhancer. However the β-Galactosidase gene used to normalise was driven by the SV40 viral promoter which is not as strong. Strong viral promoters may dilute out transcriptional machinery and affect the expression of the gene being measured. This phenomenon has been observed where the normaliser was shown to affect the expression of the vector of interest (Farr & Roman, 1992). The Promega dual luciferase manual also mentions this problem and suggests transfecting with the normalising vector at a higher ratio. Although a 1:10 ratio was used, this still gave very strong *Renilla* luciferase expression and a ratio of 50pTRR2Hyg-Luc : 1 Renilla vector could be suitable to minimise promoter cross talk effects.

The use of a PCR screening process was very successful at quickly establishing which cell lines contained the rtTA gene, and prevents the un-necessary growth and screening of cell lines lacking the rtTA gene. These cell lines can now be screened using the dual luciferase assay, except changing the protocol to using an overall low concentration of DNA in the transfection, and a much lower molar ratio of the *Renilla* control plasmid relative to the pTRE2Hyg-luc reporter plasmid. These measures should allow the confident identification of an inducible cell line. A further option in establishing a tetracycline inducible cell line is to try using the new TetOn advanced system. A common problem preventing high levels of induction is unacceptably high background levels of the gene of interest under the control of the TRE/CMV minimal promoter. The Tet-On advanced system contains a modified TRE/CMV minimal promoter which further reduces background expression increasing induction. As some consolation a colleague (Craig Smith) has also been trying to establish the Tet-On inducible system in bovine embryonic fibroblasts and out of the cell lines generated, using the luciferase screening test, he also found none of them exhibited the inducible phenotype.

CHAPTER 5 : GLOBAL ANALYSIS OF GENE EXPRESSION USING A MICROARRAY APPROACH

5.1 OVERVIEW

The Affymetrix GeneChip® Expression analysis platform was chosen to use for microarray analysis of mTS cell gene expression under different treatments. This array uses multiple 25-mer oligonucleotide probes per gene/expressed sequence synthesised at predetermined locations on a glass wafer. TS cells of equal passage number were treated for 24 hours with either Elf5 specific siRNAs which mediated *Elf5* mRNA knockdown, negative control siRNA, or TS media containing different combinations of Fgf4 and Activin/TGFβ growth factors. Total RNA from these experiments was then isolated using the TRIZOL (Invitrogen Life Technologies) method and treated with DNase (section 2.2.5.2). Samples were pooled into groups of three and labelled according to the Affymetrix GeneChip expression One Cycle target labelling protocol (section 2.2.5.4). This consisted in making double stranded cDNA from the collected RNA samples using a T7 RNA polymerase oligo dT primer. From the resulting cDNA, cRNA was produced using the T7 RNA polymerase which incorporates biotinylated ribonucleotide analogues into the RNA being produced. Labelled RNA was then hybridized to a GeneChip Mouse genome 430 2.0 Array. Hybridisation and scanning of the arrays was carried out commercially at The University of Auckland's Affymetrix facility. After hybridisation the array was stained with a fluorescent dye (SAPE) which conjugates to the biotin modified nucleotides. The array was scanned and the intensity of each probe set was measured to give a quantitative value for the expression of that gene (Affymetrix Inc., 2006a).

Array Intensity data was analysed using a robust means analysis (RMA) algorithm (GenStat software package 9th edition; VSN international ltd, UK) which gives a normalised average expression level for each gene on each array. This value was then averaged across arrays hybridised with samples of the same treatment. Comparisons were carried out between control and treated samples. Using predetermined fold change cut-offs (section 2.2.5.7.2) genes of interest were identified for each

comparison. Expression values of a selection of these genes were then later verified for change in expression using quantitative real-time PCR.

5.1.1 Microarray Technology

DNA microarrays are a powerful tool to analyse the transcriptional response of a cell to a perturbation because they allow the simultaneous measurement of thousands of transcripts at once. This facilitates the identification of physical or functional connections between gene products (Brown & Botstein, 1999). There is a tight connection between the function of a gene product and its expression pattern. Therefore by changing the expression of Elf5, the function of this protein, i.e. its down stream target genes, was hoped to be determined. Futhermore, expression profiles from microarrays hybridised with different targets can be compared and aligned allowing similar cellular responses to be identified (Brown & Botstein, 1999). Thus it was envisioned that by analysing mTS cells under several conditions which are known to affect their 'stemness' and lead to their differentiation, such as the expression of Elf5 or the presence of Avtivin/Nodal and FgF4 growth factors, common networks of genes could be identified which are involved in maintaining the phenotype of mTS cells.

Microarrays consist of a solid support surface (generally a glass slide) with thousands of 25-75mer oligonucleotide probes fixed to the surface. The DNA/RNA sample (known as the target) is fluorescently labelled and applied to the microarray slide where it binds to complementary probes (hybridisation). The oligo probes on a microarray are designed to interrogate a particular region of a target and minimise cross-hybridisation (Elvidge, 2006). They are either synthesised and mechanically spotted on to the array or, in the case of Affymetrix arrays, 25mer probe sequences are synthesised at known locations *in situ* by using light directed synthesis (Lipshutz *et al.*, 1999). This allows many more probes to be put on an array than can be mechanically spotted.

After hybridisation with the target, the array is scanned with a laser which excites the target associated fluorescent dye, giving an emission which estimates the relative amounts of different transcripts present (Allison *et al.*, 2006). The sample target applied to the array can be either cDNA or cRNA. In a cDNA microarray mRNA from samples to be compared is reverse transcribed and concomitantly labelled with either Cy3 or Cy5 fluorescent dyes. These two different labelled samples are co-hybridised to

one array and then the Cy3 and Cy5 fluorescence measured separately. A merged image is produced which gives the relative expression from each sample. In a cRNA microarray experiment cDNA is also produced from the mRNA sample, this is then converted to double stranded cDNA and then to biotinylated cRNA. In this case only a single colour of fluorescence is hybridised to each array (Allison *et al.*, 2006).

5.1.2 Technical Aspects of the Microarray Experiment

5.1.2.1 Choice of a Microarray Platform

Gene-expression measurements using hybridization technology, has become a common technique in biomedical research. A comprehensive study of the intra-platform, inter-platform and inter-laboratory reproducibility of ten different platforms available for gene-expression studies was carried out by Kuo *et al.* (2006). The results showed that the most consistent results were achieved by commercially available platforms rather than arrays generated 'in house' (Kuo *et al.*, 2006), and platforms which used one dye were more consistent than two-dye platforms. Overall the Affymetrix GeneChip® commercially produced one-dye platform appeared to perform the best and therefore this platform was chosen for the global analysis of gene expression.

5.1.2.2 Technical aspects of the Affymetrix GeneChip array

Affymetrix arrays are high density oligonucleotide (25mer) arrays and have the advantage of containing thousands more features than cDNA arrays. The high density arrays even allow a whole genome of probe sets to be analysed on one array; as is the case with the mouse 430 2.0 whole genome array used in this experiment which contains 45,000 probe sets. Eleven pairs of probes make up a probe set and are used to measure the expression of each sequence represented on the array. Therefore the mouse 2.0 array contains 495,000 features. Each pair consists of a perfect match probe (PM) and a mismatch probe (MM). The MM probe is the same as the PM probe except for a central base substitution and helps to determine the level of non-specific binding. Such a high density of features is achieved through the use of photolithography and solid phase DNA synthesis (Lipshutz *et al.*, 1999). Light is directed to specific areas on the surface of the array, this removes photolabile protecting groups at that position. Hydroxyl-protected deoxynucleotides are then

incubated with the surface and chemically attach to areas which have previously been illuminated (Lipshutz *et al.*, 1999). This process is repeated to synthesise specific DNA oligonucleotides at known locations.

The sequence of a probe set is designed to specifically monitor the expression levels of as many genes as possible. A probe set is designed from a 200bp to 300bp segment of a transcript and then non-overlapping 25mer oligonucleotides are selected. Multiple oligonucleotides of different sequences designed to hybridise to different regions of the same mRNA improve the signal to noise ratios and reduce the chance of a falsely identified differentially expressed transcript; measurements on a typical eukaryotic array put the false positive rate as 2% (Lipshutz *et al.*, 1999). The hybridisation signal intensities have been shown to be directly proportional to RNA concentration

5.1.2.3 Image processing Algorithm

After scanning of the arrays the raw fluorescent intensity needs to be converted into a numerical value which is comparable for each probe set between multiple arrays and treatments. Although there is some controversy over the best method, the method robust multi-array average (RMA) performs well or better than competitors for Affymetrix arrays (Allison *et al.*, 2006). RMA analysis involves three steps: background correction, normalisation and summarisation. The details on the RMA process are given in Analysis of Microarray Data in Genstat, pages 24-26 (Baird, 2006) In brief: the background correction removes the mean of the lowest 2% intensities; it also fits a noise model to the intensities from the perfect match cells. The normalisation corrects the intensities over the slides so that each slide has the same profile (allowing slides to be compared). Finally the summary over the probes is done. This calculates the average intensity over the 11 probes for each gene taking into account slide effects.

5.1.3 Microarray Design

A crucial aspect of microarrays is biological repeats where measurements are taken from multiple biological cases (Allison *et al.*, 2006), with technical repeats almost never required; therefore no technical repeats were included in the design. Allison *et al* (2006) also suggest a minimum number of five biological cases per group and that "pooling samples can be beneficial when biological variability is high relative to measurement error, and when biological samples are inexpensive relative to array cost" as is the case

with the experiment carried out here. A further consideration in design of the experiment was the fact that RNA interference experiments, such as the siRNA experiments performed here can induce non-specific responses (Bridge *et al.*, 2003). Samples from the two best siRNAs which knock down *Elf5* were both used, as there is a low chance that the same non-specific effects will be caused by two distinct siRNA sequences (Echeverri *et al.*, 2006). Also the siRNA control oligonucleotide from Invitrogen was used to provide a control for sequence independent effects of siRNA treatment (Invitrogen Life Technologies, , 2004b).

An experimental design was thus decided on which included three pooled mRNA samples per array and either two or three arrays were used per treatment (giving a total of six or nine samples per treatment). The number of arrays per treatment (two or three) was decided on depending on how important the information gained from each treatment was envisioned to be. Twelve wells of two six well plates were used for each treatment. Cells were of a low passage number, and were at passage 7 at the time of the experiment, although not all treatment experiments were carried out at the same time.

To avoid 'confounding extraneous factors' (Allison *et al.*, 2006) arrays used were from the same batch and all samples were processed after RNA isolation concurrently.

5.1.4 Total RNA Isolation and Quality Assessment

Total RNA was isolated using the TRIZOL method (Invitrogen Life Technologies) according to the protocol given in section 2.2.3.2 until the end of the ethanol precipitation after the DNase reaction.

Three sources of variation can affect the accuracy of microarray data: biological variation; sample preparation (RNA extraction and labelling), and system variation (instrument and array variation). Due to internal controls and standardisation of techniques system variation is negligible (Affymetrix Inc., 2006b). To minimise biological variation RNA samples should be of a high quality. To assess total RNA quality RNA after RNA extraction 1µl of total RNA sample was removed and used for reverse transcription followed by real-time PCR and a second 1µl was analysed for concentration and purity on the Nanodrop spectrometer followed by running on an agarose gel (section 2.2.5.2). Any samples which failed the quality control tests were

discarded, and in some cases experiments had to be repeated before a satisfactory set of RNA samples were obtained for each treatment.

5.1.4.1 Nanodrop Absorbance Ratio

RNA isolated should have an absorbance ratio A260/A280 of between 1.6 and 1.9 in water. Ratios below 1.6 indicate protein contamination and ratios above 2.1 indicate degraded RNA (Affymetrix Inc., 2006b). It was also desirable to have a consistent concentration of total RNA, at least above 500ng/μl. The Results from Nanodrop analysis of all RNA samples are given in the appendix.

5.1.4.2 Analysis of Total RNA Quality using an Agarose Gel

Because RNA is very susceptible to degradation, it was important to check the quality of extracted RNA before proceeding. Both ribosomal RNA bands (18S and 28S) should be clearly seen on a gel (Perkel, 2006) to ensure good RNA quality. This was done by checking the RNA on a gel (section 2.2.2.6). Below is a sample gel from some samples which were successfully isolated. As it can be seen from the gel the two ribosomal RNA bands can be clearly seen. Samples which did not have clear bands were not used for the microarray.

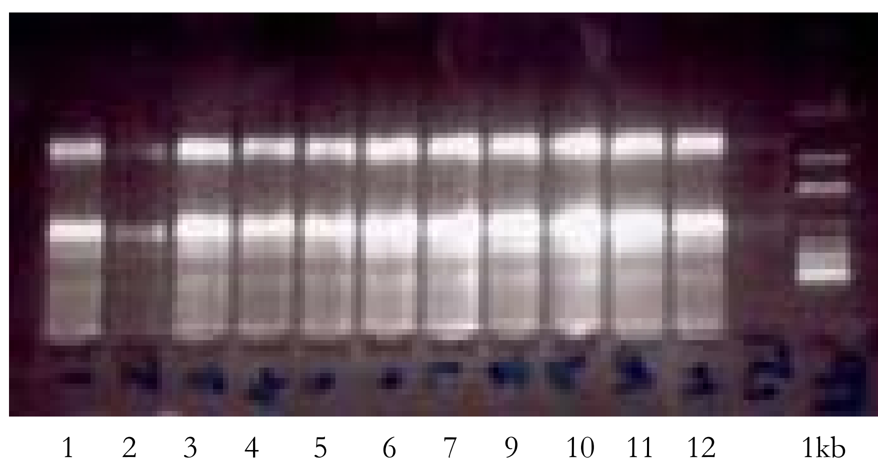


Figure 5.1-1: 1.4% agarose gel of RNA sucessfully extracted from 'Normal' treated mTSsamples. Samples are shown 1-12, and a 1kb ladder (Abgene). Two rRNA bands can clearly be seen indicating RNA is good quality.

Another technique is to use the bioanalyser as suggested by Affymetrix. A high RNA Integrity number indicates good quality RNA. During a demonstration of the Agilent 2100 Bioanalyser (Agilent Technologies Inc, 2006) five left over RNA samples which

had been used on the microarray and had subsequently been stored at -80°C for about 2 months were checked. These gave RNA Integrity Numbers (RIN) between 8.5 and 9.6 (out of a maximum value of 10). This shows the RNA used was of very good quality.

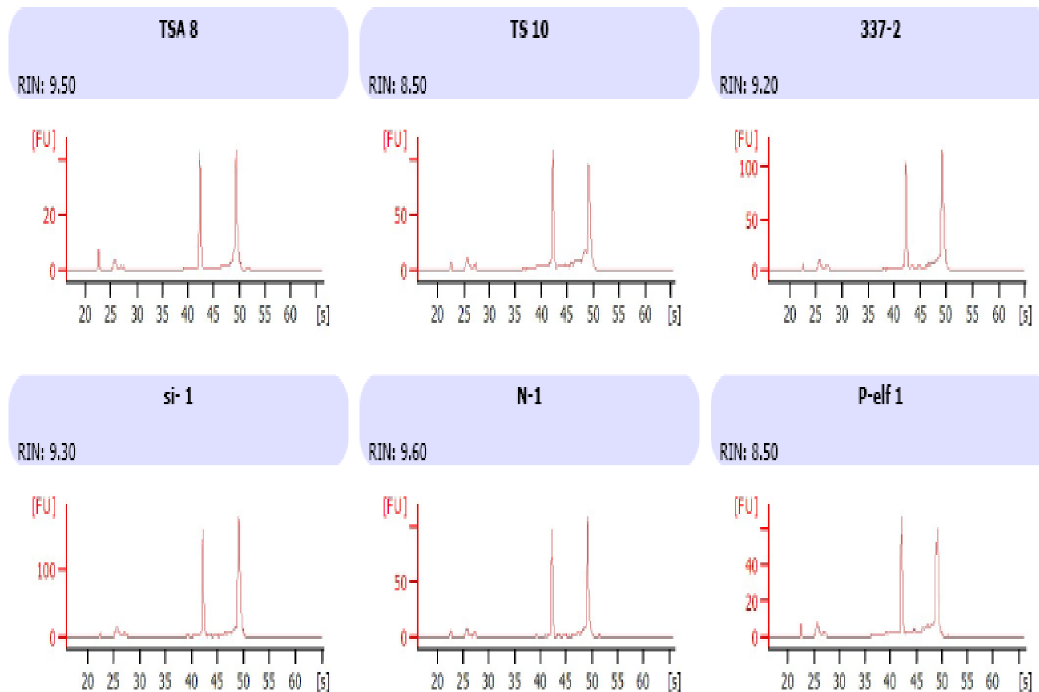


Figure 5.1-2: Analysis of a few of the total RNA samples used in the microarray experiment using the Bioanalyzer (Agilent Technologies Inc, 2006). An RIN value ≥8 is acceptable for proceeding to cDNA synthesis. TSA= TS cells grown in Trophoblast medium supplemented with Activin; TS= TS cells grown in trophoblast stem cell medium containing no added growth factors; 337= TS cells treated with siRNA 337; N=TS cells grown in normal growth medium containing Fgf4 and Activin growth factors; P-elf = TS cells transfected with a pTracer-Elf5 construct.

5.1.4.3 Analysis of Gene Expression as a quality check

As described in section 2.2.5.2 1µl of each total RNA sample (12 samples per treatment) was reverse transcribed and the resulting cDNA analysed by real-time PCR using the ABI realtime PCR machine. Real-time PCR analysis allowed three parameters of the samples to be checked:

i. DNA contamination of the total RNA sample by measuring the Gapdh Ct of each cDNA sample and comparing it to the Gapdh Ct obtained from using a 1/20 dilution of the total RNA sample (not reverse transcribed).

ii. The normalised copy number of *Elf5* mRNA was measured in the siRNA treated samples to ensure successful knockdown had occurred as compared to

the siRNA negative control samples. Also from previous experiments it was known that *Eomesodermin* expression is very sensitive to changes in the availiability of growth factors. Therefore to check the samples grown without F4H/Activin had responded in the predicted manner and that control cells still maintained a higher expression of *Eomesodermin* the normalised expression of *Eomesodermin* was evaluated.

iii. The raw Gapdh Ct was used as a check of the overall quality of the samples and to check there was minimal variance between samples indicating a consistent RNA extraction. A low Gapdh Ct could indicate RNA had been lost or degraded during the RNA extraction.

For each treatment 12 samples were collected and analysed as described by real-time PCR. An example of the normalised *Eomesodermin* expression relative to the average 'Normal' expression is shown in

iv. Figure 5.1-3 for three samples each of TS plus activin, TS with Fgf4 and Normal media containing conditioned medium and Fgf4. The pattern of *Eomesodermin* expression was as expected; it was reduced in the samples grown without growth factors. The relative expression of *Elf5* and *Eomes* for all samples used in the microarray is given in the appendix A-3.

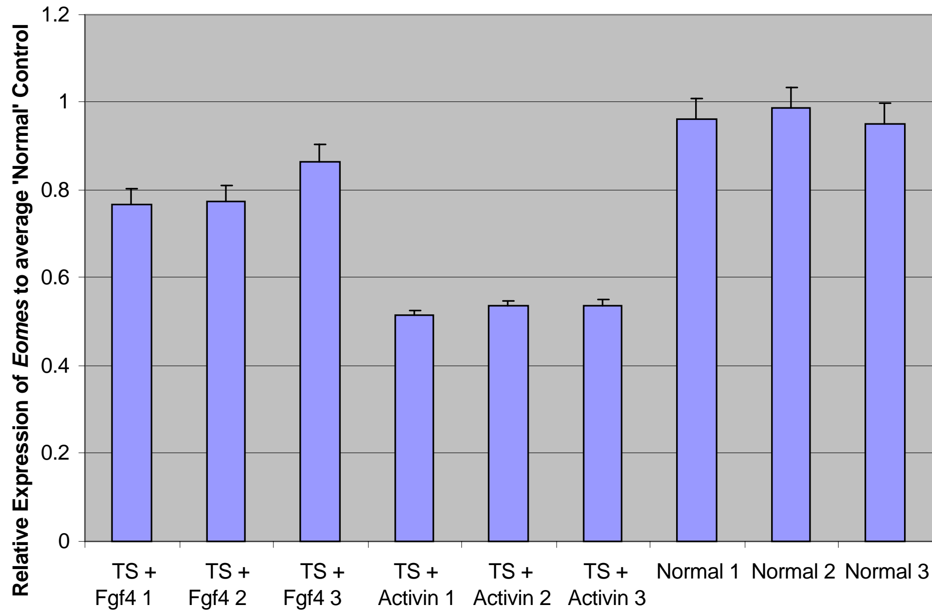


Figure 5.1-3: Relative expression of *Eomesodermin* was measured using real-time PCR and used to assess samples were suitable to use in the microarray experiment. The RNA isolated from cells grown without activin or Fgf4 growth factors should have a lower expression of *Eomesodermin* relative to the cells grown in Normal growth media. *Eomesodermin* expression was normalised to Gapdh expression and is shown relative to the average expression of the normal samples (+/- sem, n=3). Samples did follow the expected pattern of *Eomesodermin* expression. TS + F4H= TS cells grown in TS medium with Fgf4; TS+ Activin=TS cells grown in TS medium with Fgf4; Normal= TS cells grown in normal medium containing both Fgf4 and Activin.

The expression of *Elf5* was measured in samples treated with 337 or 733 siRNA and was calculated relative to the expression of siRNA control treated samples. Figure 5.1-4 shows the relative *Elf5* expression for the first three samples from these treatments. *Elf5* knockdown appeared greatest in 337 siRNA treated samples and was about 80%, 733 siRNA knockdown was on average 62%. Although the knockdown in 733 treated samples was not optimal the main purpose for using the second siRNA oligo was to verify the results from the first oligo and due to time constraints it was decided to use these samples. The non-reverse transcribed (RT-) *Gapdh* Ct of all samples used for labelling in the microarray was always greater than six Ct units (in general it was about 10 Ct units greater) than the raw *Gapdh* Ct indicating very little DNA contamination in the samples. The raw *Gapdh* Ct was within 1 Ct unit between samples used for labelling, indicating samples had a similar relative abundance. A table showing all of the quality control parameters for all samples able to be used for the microarray is given in the Appendix A-3.

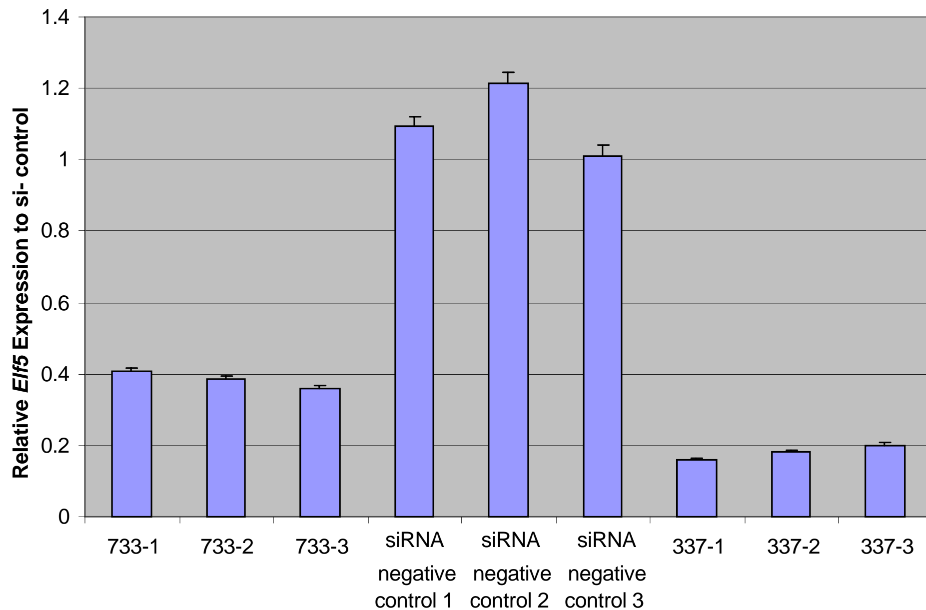


Figure 5.1-4: Relative expression of *Elf5* was measured in siRNA treated samples to assess samples were suitable for use in the microarray experiment. *Elf5* expression was normalised to *Gapdh* expression and is shown relative to the average *Elf5* expression of siRNA negative control treated samples (+/- sem, n=3) Knockdown was greater than 80% in samples treated with 337-siRNA, and greater than 60% in 733 treated samples. 733=siRNA 733 treated samples; 337=siRNA 337 treated samples.

5.1.5 Labelling of RNA samples for the microarray

5.1.5.1 Pooling of RNA samples

Samples were pooled into groups of three based on their RNA concentration (as measured on the Nanodrop). Pooling was decided on in the microarray experimental design to allow the total number of samples tested to be higher. However pooling samples produces a signal for a gene which is like the arithmetic mean of the samples; one sample could bias the pooled sample (Affymetrix Inc., 2006b). To minimise the influence of a single sample on the pool equal quantities of RNA from each sample were added to the pool. This was done by choosing three samples which had passed all of the quality checks and had similar concentrations of RNA.and using equal volumes of each. The individual volumes were calculated to give an overall concentration of 10µg of RNA per pooled sample. The total volume was made up to 8µl with RNAse

free water so each sample was ready for the first labelling step. An example of the amounts used in the first array is shown below:

Table 5.1-1: Example of the spreadsheet used to calculate the volumes of each sample used for each pool.

Normal treatment **slide 1**	sample number	Conc. of sample (*ng/ul*)	*ng* per sample
	2	2372	3293
	3	2431	3375
	11	2400	3332
	average conc. (ng/ul)	2401	
	Total (ng)		10000
volume per sample (ul)	1.39	volume H20 added (*ul*)	3.84

5.1.5.2 Labelling of Pooled RNA samples

Pooled RNA samples were labelled as described in section 2.2.5.4. The labelling process involved creating cDNA by using a T7 oligo dT primer which hybridises to the poly-A tail of mRNA. After the first strand of cDNA was made a second DNA strand was produced to give double stranded DNA. Following ds-cDNA production two samples were run on an agarose gel to check the size distribution of the cDNA products. As is can be seen from the gel (Figure 5.1-5) the cDNA ranged in length from about 250bp to 3000bp.

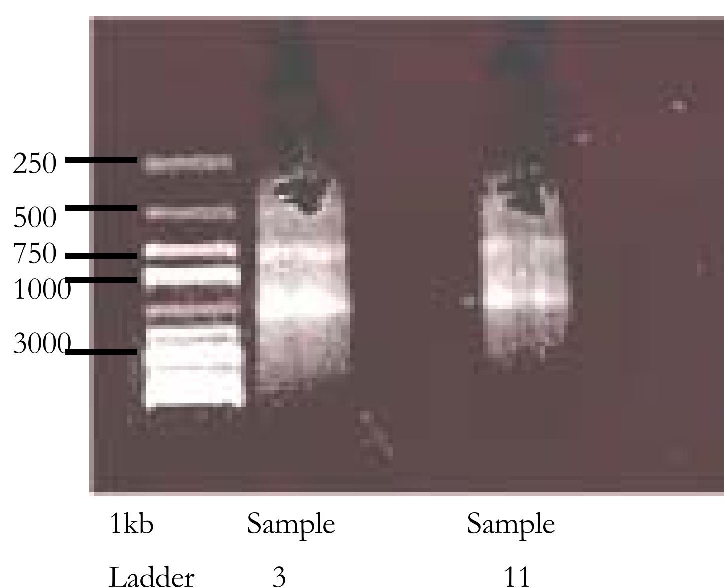


Figure 5.1-5: ds-cDNA during labelling. 1µl of samples 3 and 11 was run on an agarose gel after the clean up step following second DNA strand synthesis. A 1kb Abgene ladder was also run and selected bands are labelled.

Following cDNA production the ds-cDNA was used to produce cRNA, using biotin labelled ribonucleotides. This was then purified using a column. An aliquot of Biotin labelled RNA from each sample was diluted 1/100 and then analysed using the nanodrop for concentration and purity. The absorbance ratio should also be between 1.6 and 1.9 for pure RNA in water. The concentration of cRNA had to be adjusted to take into account unlabelled total RNA. A 100% carryover was assumed and the cRNA yield was calculated using

Adjusted cRNA yield = RNAm-(total RNAi)(y) Equation 6 (section 2.2.5.4.6).

The concentration, purity and adjusted cRNA yield of the biotin labelled cRNA is given in Table 5.1-2.

Table 5.1-2: Calculating the adjusted cRNA yield. The purity and concentration of the biotin labelled cRNA measured using the Nanodrop spectrometer. The adjusted cRNA yield was then calculated assuming a 100% carryover of total RNA. The adjusted yield was equal to the measured amount of cRNA -10µg x 0.5, since the starting amount of total RNA was 10µg and half of the cDNA produced from this was used to make the biotin labelled cRNA.

Pooled Sample number	nanodrop measurement ng/ul	Absorbance Ratio 260/280	x100 (dilution) ug/ul	x 19ul ug cRNA	adjusted yield (-10x0.5) ug cRNA
1	46.2	1.61	4.62	87.78	82.78
2	51.3	1.62	5.13	97.47	92.47
3	41.8	1.61	4.18	79.42	74.42
4	44.2	1.63	4.42	83.98	78.98
5	41.1	1.67	4.11	78.09	73.09
6	39.7	1.6	3.97	75.43	70.43
7	47.1	1.64	4.71	89.49	84.49
8	47.5	1.66	4.75	90.25	85.25
9	49.3	1.65	4.93	93.67	88.67
10	45.4	1.65	4.54	86.26	81.26
11	39.1	1.63	3.91	74.29	69.29
12	42.9	1.62	4.29	81.51	76.51
13	35.3	1.62	3.53	67.07	62.07
14	40.6	1.65	4.06	77.14	72.14
15	33.5	1.64	3.35	63.65	58.65
16	33.1	1.62	3.31	62.89	57.89
17	44.7	1.59	4.47	84.93	79.93
18	49.9	1.62	4.99	94.81	89.81

Twenty micrograms of the cRNA (calculated using the adjusted yield) was fragmented as described in section 2.2.5.4.7. An aliquot of fragmented and un-fragmented cRNA

was run on agarose gel to check the fragmentation had occurred successfully. The fragmentation process should result in a distribution of RNA between 35 and 200 bases. The gel is shown in figure Figure 5.1-6. The unfragmented cRNA was distributed mostly between 250 and 3000bp in length. Similar to the distribution of cDNA shown in Figure 5.1-5, however the fragmented cRNA was all smaller than 250bp long showing fragmentation had occurred successfully. Following fragmentation samples were added to a hybridisation cocktail (section 2.2.5.5). The hybridisation cocktail was taken to the Affymetrix facility at Auckland University and the hybridisation, washing and scanning was carried out there by Liam Williams following the standard protocol (section 2.2.5.6).

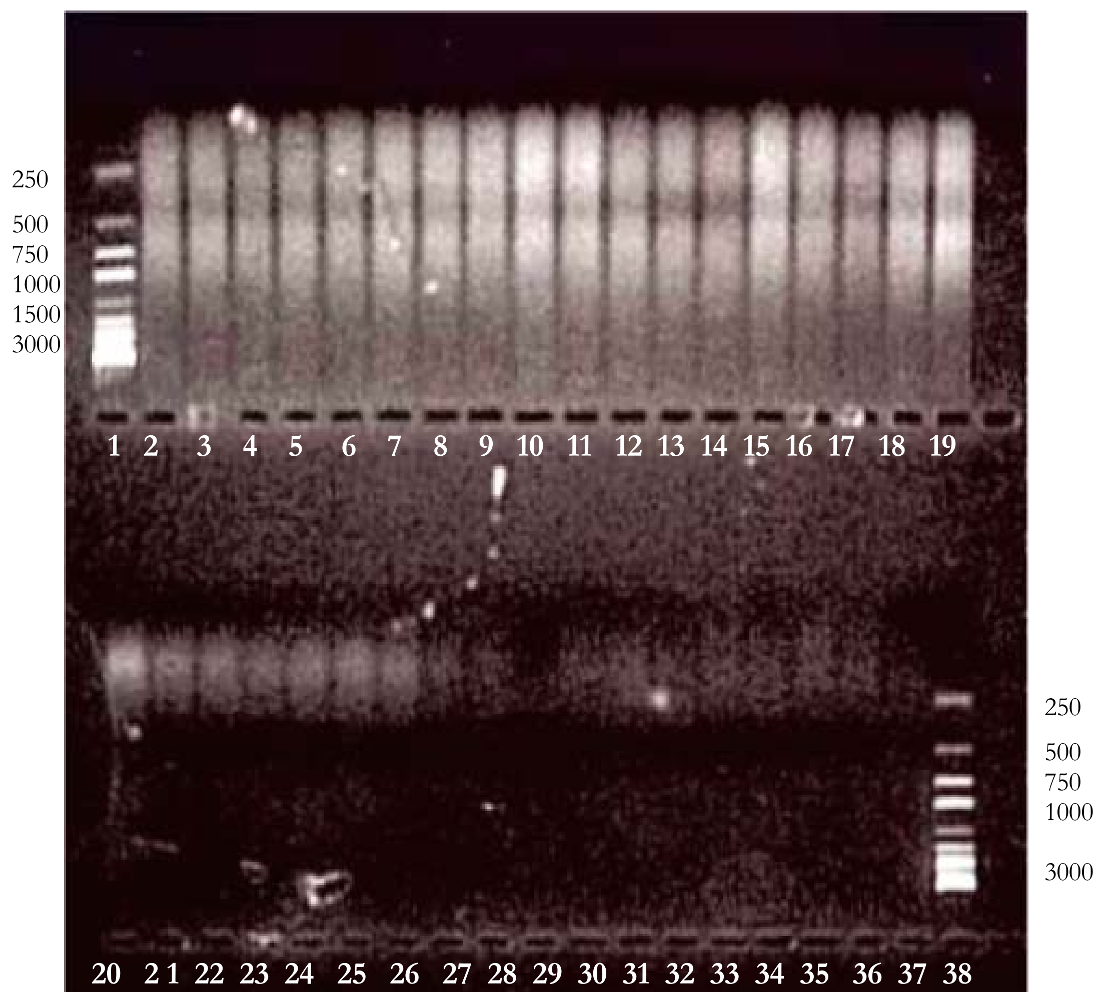


Figure 5.1-6: Non-fragmented and fragmented cRNA. 1.7% agarose gel showing lanes 1 and 38 Abgene 1kb ladder (UK), lanes 2-19 un-fragmented cRNA (~1.5µg cRNA per lane); lanes 20-37 fragmented cRNA (~1µg cRNA per lane). Fragmented cRNA was mostly smaller than 250bp.

5.1.6 Microarray Analysis

Following washing and scanning (section 2.2.5.6) of the arrays the raw intensity data was analysed as detailed in section 2.2.5.7. Briefly the intensity data was read from the Affymetrix GCOS software v1.4 (Affymetrix Inc. 2005) into the GenStat statistical software program (9th edition, VSN International Ltd, UK) and an RMA analysis performed. The average $\log_2$ intensity value for each probe set was used to perform a single channel ANOVA to find significant differences between the average expression intensity of a treatment and its appropriate control. The growth factor experiments were all compared to the 'Normal' treated samples, while the Elf5 siRNA treated samples were compared to the siRNA negative control slides. A contrast between the siRNA negative control treated samples and the Normal treated samples was also performed as a quality control measure to identify genes which may be significantly changed simply due to siRNA treatment. The contrast values, their significance (*p values*) as well as the $\log_2$ intensity data for every probe set were exported to Excel for identification of target genes.

5.1.7 Quality Control of Microarray Data

Several quality control parameters were investigated following the advice given in the Affymetrix data analysis fundamentals manual. A brief description of each parameter is given below along with the result.

5.1.7.1 Probe Array image Inspection

The .dat files were visually inspected to check for image artifacts such as scratches or bubbles, or a high background. All of the JPG image files looked OK, except for a criss cross pattern. Liam at Auckland University was contacted regarding this observation. He stated that this pattern was simply a display anomaly (Liam Williams, personal communication).

5.1.7.2 B2 Oligo Performance

The Hybridisation cocktail had been spiked with the B2 control Oligo. This labelled RNA oligo should hybridise to the slide in a checkerboard pattern and it should also hybridise in a pattern which reveals the chip name (as seen in Figure 5.1-8).

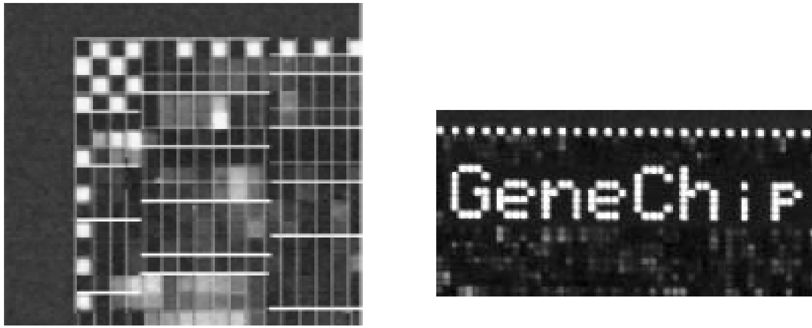


Figure 5.1-7: Hybridisation of the B2 oligo gave the correct checkerboard pattern and the chip name pattern.

The background value is a measure of the signal intensity caused by autoflourescence of the array surface and non-specific binding of the target or fluorescent stain molecules. A high background reduces the sensitivity of the experiment. Affymetrix suggests a background value of between 20 and 100. As can be seen from Table 5.1-3 the background was always within this range for all arrays.

The noise is a measure of pixel to pixel variation due to electrical noise of the scanner and sample quality. Noise values should be similar for all the arrays. From the table below all arrays appear to have similar levels of noise.

Table 5.1-3: Background flourescence and noise vlaues for the 18 microarray slides. Background should be lower than 100 and noise values all similiar between arrays.

	Background		Noise	
Array number	Average	Standard Deviation	Average	Standard Deviation
1	69.75	1.02	3.69	0.09
2	65.42	0.78	3.81	0.46
3	64.52	1.04	3.13	0.07
4	59.2	0.8	3.2	0.07
5	58.81	0.87	2.96	0.08
6	67.65	0.85	3.34	0.07
7	61.64	0.89	3.35	0.1
8	60.97	0.75	3.18	0.09
9	63.08	0.72	3.34	0.06
10	57.66	0.83	3.04	0.1
11	57.93	0.82	3.19	0.06
12	60.01	0.47	3.11	0.06
13	60.6	0.78	2.95	0.08
14	66.96	1.02	3.32	0.06
15	62.12	0.59	3.48	0.07
16	64.56	0.66	3.29	0.08
17	71.13	1.14	3.71	0.07
18	67.74	0.88	3.37	0.05

5.1.7.4 Poly-A Controls

Poly-A RNA controls were spiked in at the beginning of the labelling process. These controls are five *B. subtilis* genes which have been modified by the addition of a poly-A tail. The poly-A controls were spiked in to each 10µg total RNA starting sample and contain known concentrations relative to the total RNA population:

Poly-A control gene	Relative Concentration
lys	1:100000
phe	1:50000
thr	1:25000
dap	1 in 7500

All of the Poly-A controls should be present with increasing signal intensity according to the ratios spiked in, i.e. *lys* smallest, *dap* the largest. An example of the expression profile from the poly-A controls is shown in Figure 5.1-8.

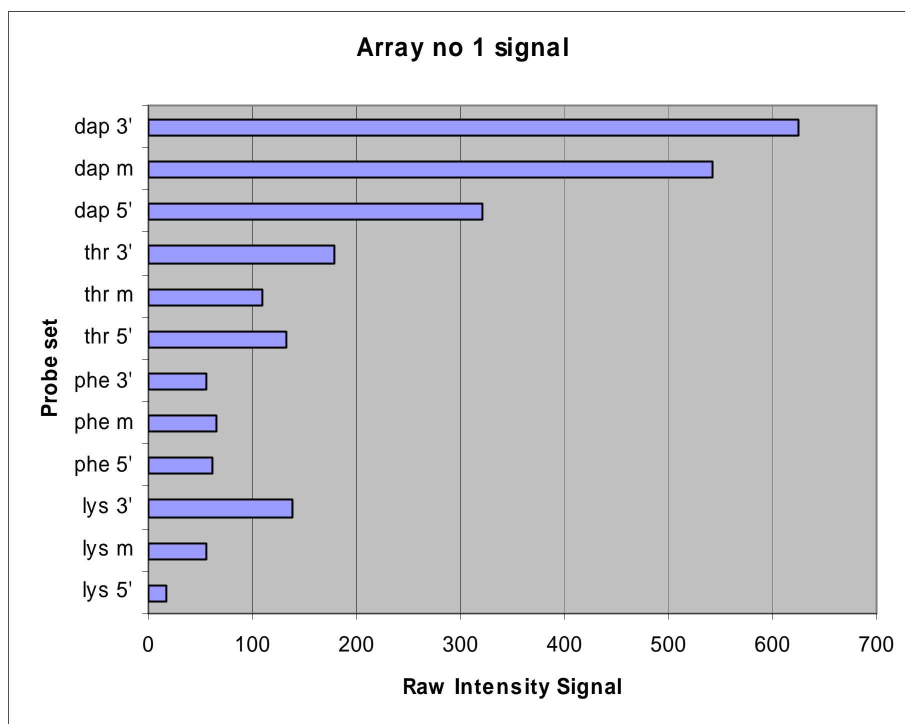


Figure 5.1-8: Raw intensity signal of the spiked in poly-A controls. The first array is shown as an example. Three probe sets existed for each poly-A control gene, a 5', 3' and m probe set which measure expression of that particular area of the gene. The Poly A controls were present in the correct respective order.

5.1.7.5 3'/5' Ratio of Housekeeping Genes

The ratio of the 3'/5' intensity of the housekeeping genes, actin and Gapdh can be used to measure the number of cDNA synthesis reactions which went to completion. Reverse transcription synthesises cDNA starting from the 3' end of an mRNA and ends at the 5' end. If all cDNA reactions went to completion then the 3'/5' ratio would be 1, however this doesn't occur and the 3' intensity is larger. Affymetrix recommends a 3'/5' ratio smaller than 3. Table 5.1-4 shows the 3'/5' ratio for *β-actin* and *Gapdh*. In all slides this ratio was less than 3; surprisingly the *Gapdh* 3'/5' ratio was smaller than one indicating that the intensity for the 5' probe was greater than the 3' probe. This could be due to a labelling bias.

Table 5.1-4: 3'/5' ratio for βactin and Gapdh; two housekeeping genes. A ratio less than 3 is acceptable as it indicates cDNA synthesis reactions went mostly to completion. Interestingly the Gapdh ratio is smaller than 1 indicating the intensity measured for the 5' probe was greater than for the 3' probe.

Array Number	*β actin* 3'/5'	*Gapdh* 3'/5'
1	1.35	0.82
2	1.37	0.82
3	1.42	0.83
4	1.44	0.82
5	1.46	0.83
6	1.50	0.80
7	1.46	0.84
8	1.70	0.90
9	1.81	0.89
10	1.75	0.93
11	1.76	0.82
12	1.55	0.82
13	1.80	0.86
14	1.83	0.85
15	1.50	0.85
16	1.79	0.85
17	1.42	0.80
18	1.47	0.81

5.1.7.6 Percent Present

The expression of a probe should be called 'present' at least 35% of the time, however this factor depends on the biological sample, i.e. which genes are being expressed. A % present lower than 35% can mean poor sample quality. As shown in the table below the % present was between 46.2-52.5% for all slides. The 'present' call for a probe set

is determined by the GCOS software and depends on the background and a number of statistical factors.

Table 5.1-5: Percentage of probes called present by the Affymetrix GCOS software. A % present lower than 35% can indicate poor sample quality.

Array Number	Treatment	% Present
1	Normal	49.13
2	Normal	47.4
3	Normal	50.8
4	TS + activin	51.7
5	TS + activin	49.8
6	TS + F4H	49.8
7	TS + F4H	51
8	TS Only	50.2
9	TS Only	46.8
10	TS Only	48.1
11	si negative	50.7
12	si negative	52.5
13	si negative	48
14	337 siRNA	46.1
15	337 siRNA	48.8
16	337 siRNA	48
17	773 siRNA	49.1
18	773 siRNA	47.2

5.1.7.7 Spiked cRNA Controls

BioB, bioC and *bioD* are genes of the biotin synthesis pathway in E. coli, and cre is the recombinase gene from the p1 bacteriophage. These were supplied as biotinylated cDNA controls which were spiked into the hybridisation cocktail just prior to hybridisation, washing and scanning of the arrays. The cRNA controls were supplied as a premixed solution at varying concentrations (GeneChip® Hybridisation Control Kit; Affymetrix Inc, California, USA):

	final concentration pM
bioB	*1.5*
bioC	*5*
bioD	*25*
Cre	*100*

BioB is at the level of detection (1:100,000 complexity ratio) and should be called present 50% of the time. In this experiment *bioB* was called present in all of the arrays.

Figure 5.1-9 shows the raw intensity values for the cRNA spiked in controls for the first array. The cRNA controls were present in the correct ratios relative to each other.

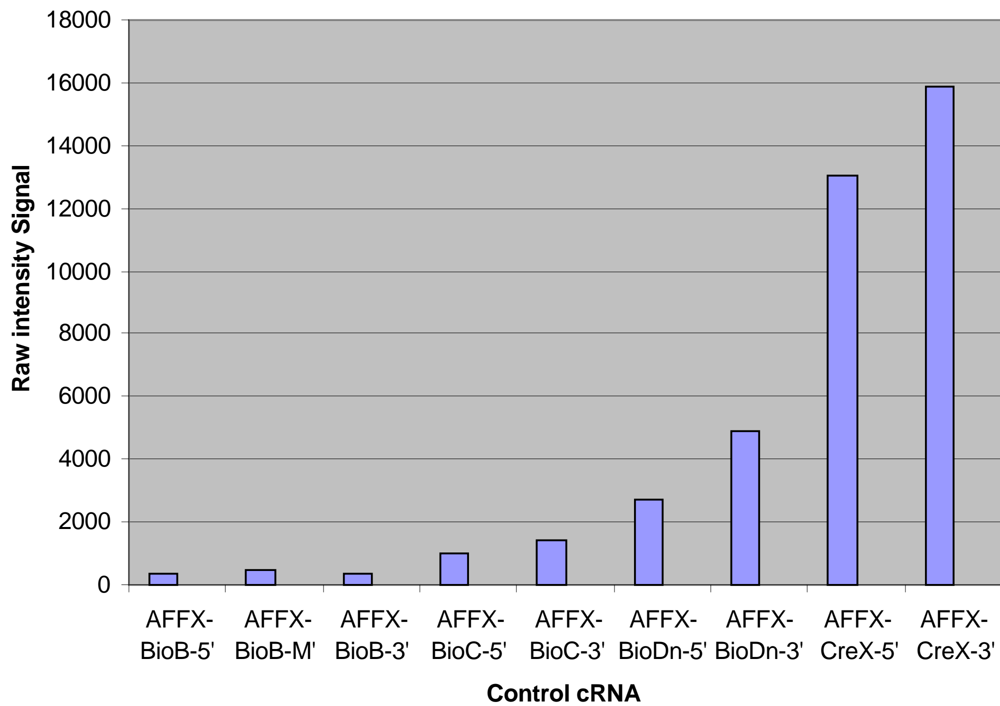


Figure 5.1-9: Raw Intensity signal for the spiked cRNA controls, spiked in at the hybridisation cocktail stage just prior to hybridisation, washing and scanning of the array for array #1. The controls show the correct ratios

5.1.7.8 Scaling Factor

To compensate for differences in the overall intensity for each array due to factors independent of transcript abundancy, such as pipetting error, hybridisation efficiency and staining efficiency the global array intensity for each array was scaled automatically by the GCOS software. This scaling factor can give an indication of low quality samples and should not differ by more than 3 fold in arrays to be compared. The largest difference in scaling factor was about 2 fold, so within the limits recommended by Affymetrix. Another way of judging the scaling performed on each array by GCOS is to look at the intensity of the cRNA controls added. Because these are independent of RNA concentration, their intensity reading should be equal in all arrays, however in bad arrays they would be scaled higher. As it can be seen in Table 5.1-6 at the measurement from the 5' cre cRNA control is closely related to the global scaling factor. The arrays with the greatest level of scaling are arrays 13, 14 and 16.

Table 5.1-6: Scaling factor and 5' *cre* cRNA control intensity measurements for the arrays. A high scaling factor indicates a poor quality sample, *cre* measurement is independent of RNA quality and directly related to the scaling factor; so a high cre measurement also indicates larger scaling.

Array number	Treatment	Scaling factor	5' *cre* cRNA Intensity
1	Normal	1.88	15877
2	Normal	2.32	16017
3	Normal	1.71	16956
4	TS + activin	1.73	16943
5	TS + activin	2.37	19843
6	TS + F4H	2.04	15845
7	TS + F4H	2.41	17500
8	TS Only	1.96	19083
9	TS Only	2.88	20248
10	TS Only	2.87	21659
11	si negative	2.39	20401
12	si negative	1.79	18900
13	si negative	3.55	23901
14	337	3.74	21395
15	337	2.65	20858
16	337	2.94	25451
17	773	2.14	18671
18	773	2.79	17302

5.1.8 Global Analysis

As shown in Table 5.1-5 above, the percent of probes called present was about 50% for all arrays. There was no obvious increase or decrease in this value based on treatment indicating the treatments did not have a large overall effect on gene expression levels.

Using Data Desk software programme (v 6.1, 1999, Data description Inc, NY) the Log_2 expression levels for 337 (Figure 5.1-10) and 733 (Figure 5.1-11) *Elf5* siRNA treatment were plotted against siRNA negative control expression levels for every probe set. The overall correlation was 0.998 and 0.994 respectively; indicating the expression levels of the vast majority of probe sets remained unaltered by the treatments; however 773 treatment had a greater effect than 337 treatment.

The same procedure was done for the average Log_2 expression for each probe set to look at the overall correlation between the normal control cells (grown with Fgf4 and conditioned medium) and those grown in the absence of Fgf4 but supplemented with activin (TS + A; Figure 5.1-12), or those grown in the absence of activin but

supplemented with Fgf4 (TS+F; Figure 5.1-13), or finally those grown in the absence of both growth factors (TS; Figure 5.1-14). The correlations were 0.991, 0.996 and 0.983 respectively. This also indicates that over the twenty four hour period of treatment there was little change in overall gene expression; however on the whole Fgf4 depletion had a greater effect than activin depletion, and removal of both growth factors had the largest effect as expected. The correlations also show that Fgf4 removal and both growth factor removal had a greater effect on gene expression than the siRNA treated samples. A scatterplot was also created between the siRNA negative control treated cells and the normal non-treated cells (Figure 5.1-15). The correlation was 0.995, indicating there was little effect on gene expression due to the siRNA control oligo or the transfection procedure.

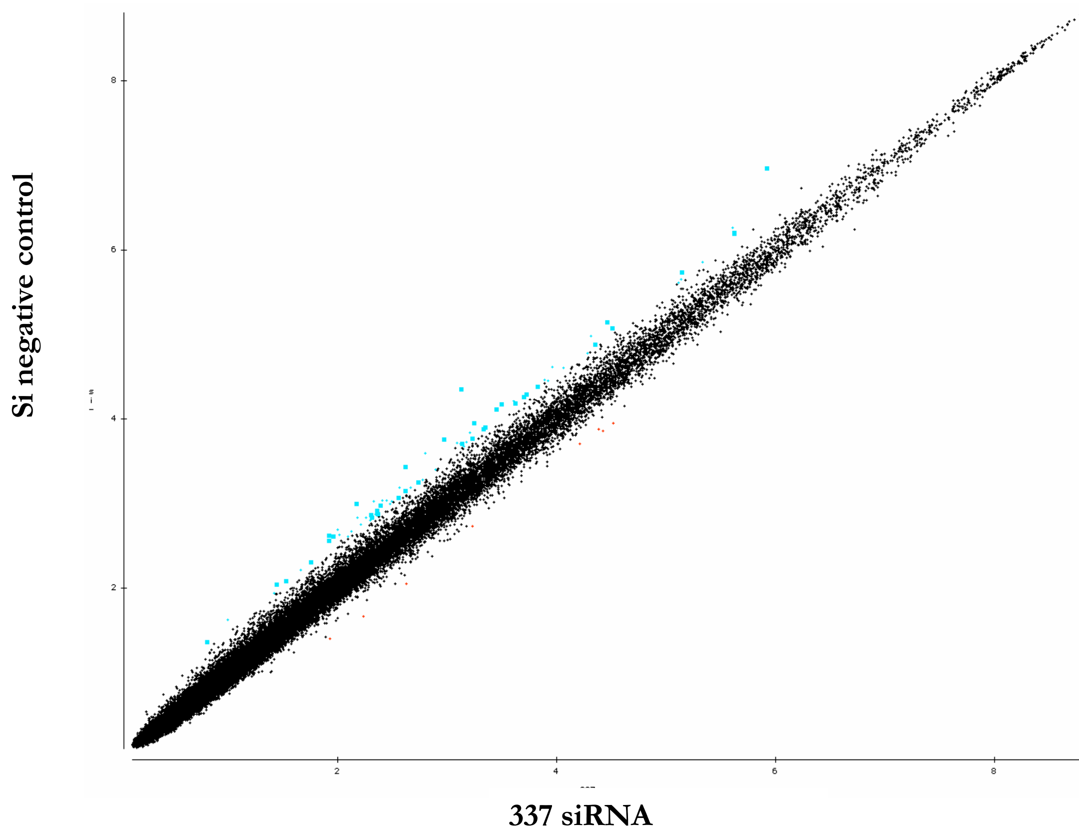


Figure 5.1-10: Scatter-plot of the siRNA negative control Log2 expression values versus 337 siRNA treated samples. R=0.998

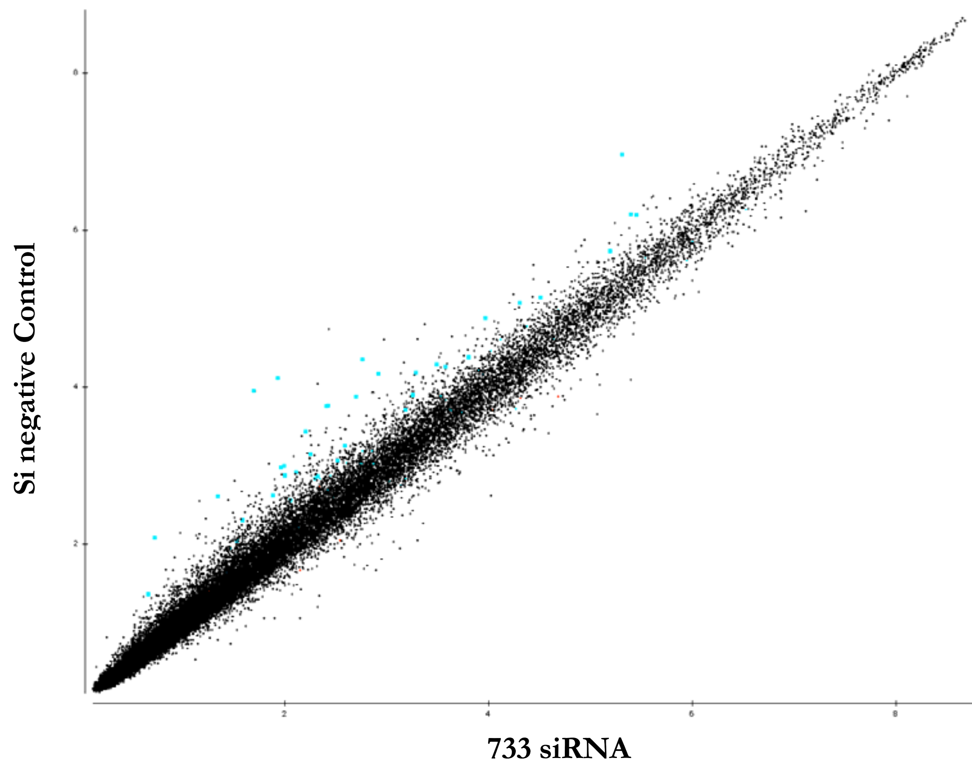


Figure 5.1-11: Scatter plot showing the overall correlation (R=0.994) between siRNA negative control and 733 siRNA Log2 expression values

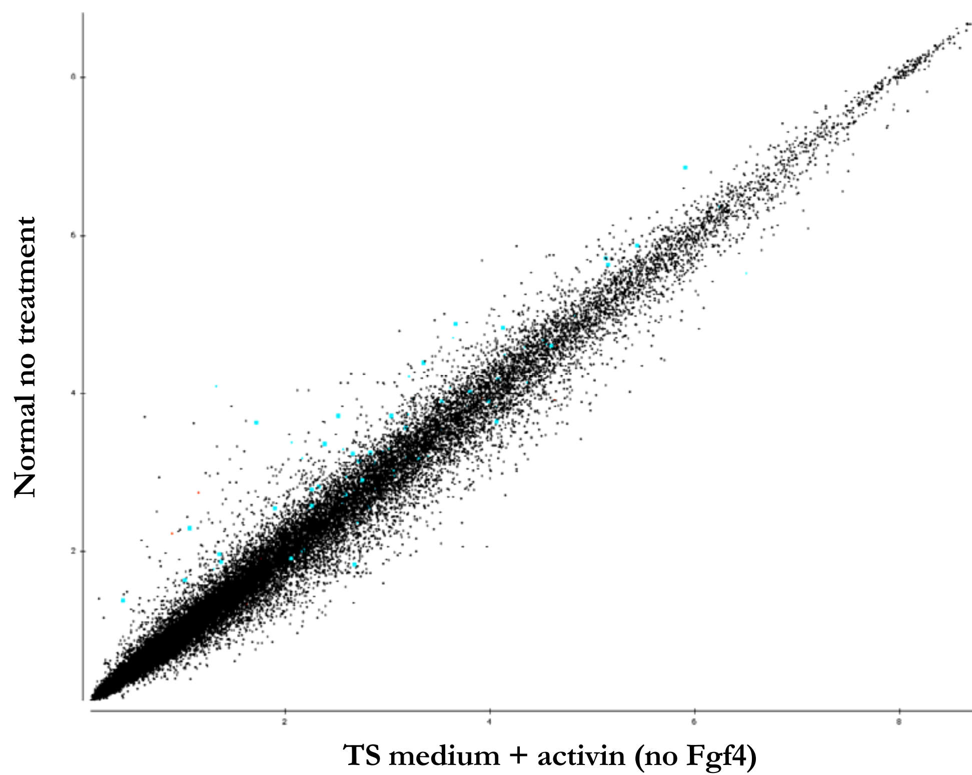


Figure 5.1-12: Scatter plot showing the overall correlation (R=0.991) between the normal growth medium control and TS medium with activin Log2 expression values

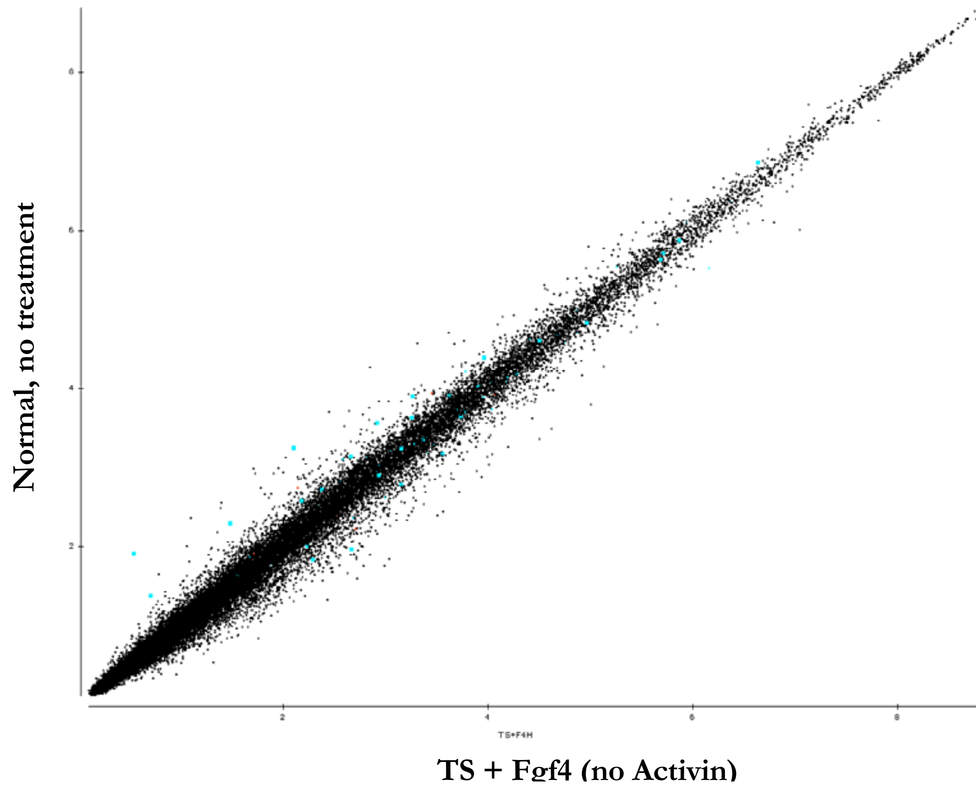


Figure 5.1-13: Scatter plot showing the overall correlation (R=0.996) between the normal groth medium control and TS medium + Fgf4 Log2 expression values

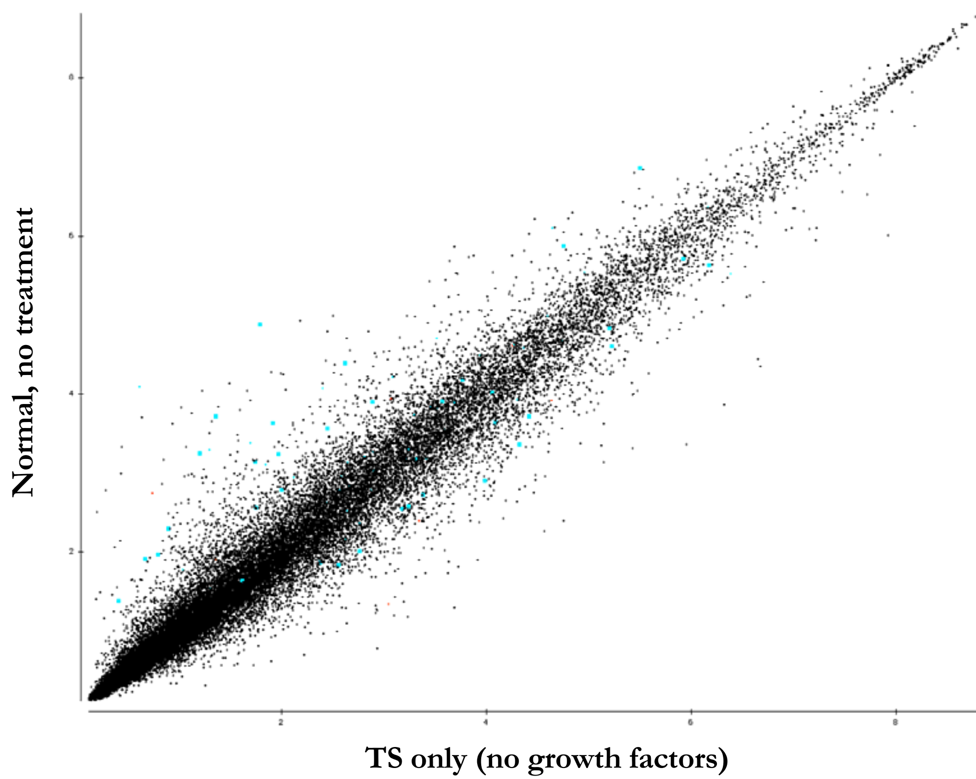


Figure 5.1-14: Scatter plot showing the overall correlation (R=0.983) between normal growth medium control and TS medium (without Fgf4 or Activin) Log2 expression values

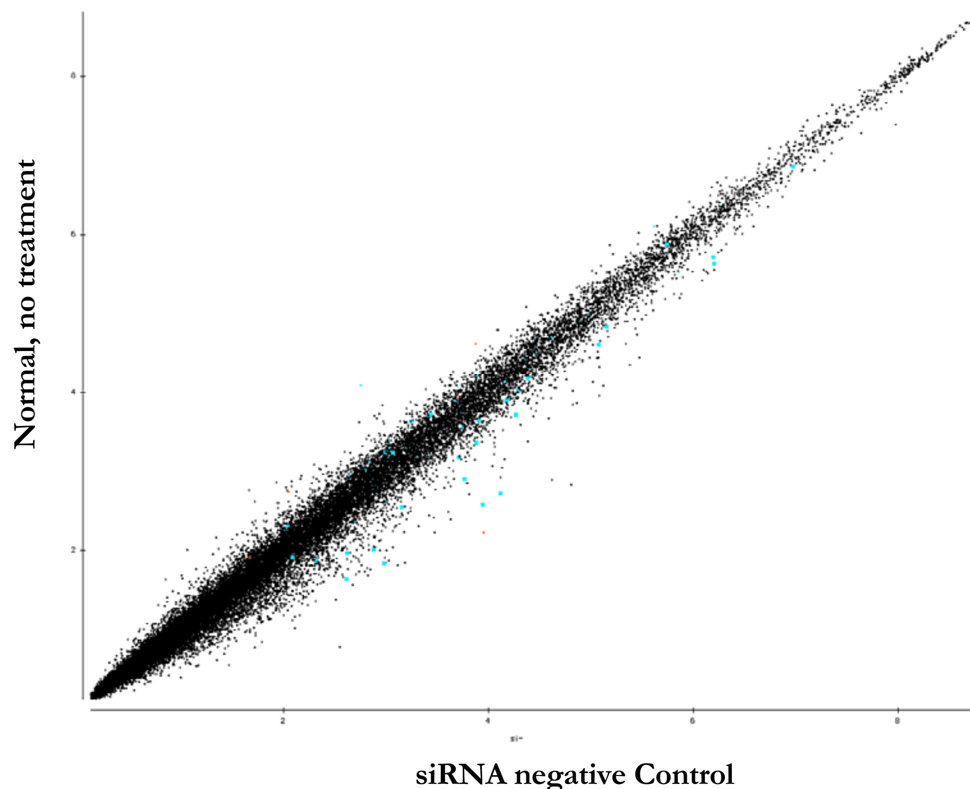


Figure 5.1-15: Scatter plot showing the overall correlation (R=0.995) between normal growth medium control and siRNA negative control Log2 expression values

Arbitrary fold change cut-offs were chosen to obtain a workable set of genes. At first a fold change cut of 2 was chosen, as a 50% change in expression of the transcription factor *Oct3/4* in ES cells has been shown to cause upregulation of mesoderm specific genes in only 24 hours (Niwa *et al.*, 2000). This shows small fold changes in gene expression can lead to significant changes in downstream gene expression and alteration of cell fate. However for the siRNA treated samples the fold change cut off had to be lowered as the maximum fold change for *Elf5* was just over 2-fold for the 337 siRNA treated samples and 3-fold for the 733 siRNA treated samples (Table 5.1-8); hence any down stream genes would be expected to have a smaller change than this. To obtain a suitable number of genes the 337 cut off was set at 1.4 fold and the 733 cut off was 1.5 fold. The fold change cut off for the growth factor arrays (removal of either Fgf4 or Actvin, or both) was kept at 2 fold. However for the list of target genes that were affected by changes in growth treatment only, the cutoff was increased to 3.5 fold.

The total number of probe sets identified as differentially expressed were combined and then duplicate probe sets removed; the resulting target set consisted in 1209 unique probe sets. To correct for non-specific effects due to siRNA treatment alone probe sets which showed a greater than 1.5 fold change in expression between the siRNA control treated samples and the non-treated ('normal') sample were removed from the sets of genes identified as differentially expressed using *Elf5* siRNA treatment, 134 probe sets were removed. The number of probe sets identified for each treatment and the fold change cut off used is shown in

Table 5.1-7 for each treatment comparison. In general all the treatments appeared to have a predominantly negative effect on gene expression, with 68% of probe sets were regulated. A total of 92 probe sets were identified as differentially expressed for treatment with the 337 siRNA compared to the siRNA control, of which 12 showed significant control siRNA: Normal differences leaving 80 probe sets. Treatment with the siRNA 733 resulted in 391 probe sets as differentially expressed. From this set 122 were removed as possibly being affected by siRNA treatment alone. The greater number of probe sets identified due to 733 siRNA treatment compared to the siRNA control reflects the greater knock-down of Elf5 expression seen in these samples; however it could also be influenced by the fact that the 733 samples were collected using a cell line grown much later. Although mTS cells were always grown under the same conditions and the same passage number was used, the fibroblast conditioned medium cells were grown in is inheriently variable (Hay *et al.*, 2004). The 337 and siRNA negative control samples were collected using the same lot of cells grown in the same conditioned medium. However the 733 cells were grown later and in different conditioned medium, so the change in expression of some probe sets could simply be reflecting this fact. To counteract this the 'normal' treated samples were collected and grown concurrently with the 733 cells, so the probe sets identified as different in the normal vs siRNA control samples and removed should remove this effect.

The number of probe sets identified as changing their expression also varied depending on the growth factors removed. Removal of Fgf4 growth factor had a much larger effect on gene expression than removal of Activin, however as expected removal of both growth factors had the greatest effect.

Table 5.1-7: Fold change cut off and the number of probe sets identified for each treatment comparison in the microarray experiment.

Contrast	Fold change cut off	Number of probe sets identified	Upregulated	Down-regulated
337 siRNA vs siRNA control	1.4	80	5	75
733 siRNA vs siRNA control	1.5	269	87	182
TS medium + activin (no Fgf4) vs Normal medium	2.0	182	71	111
TS medium + Fgf4 (no activin) vs Normal medium	2.0	28	5	23
TS medium (no growth factors) vs Normal medium	2.0	516	173	343

5.1.9 *Elf5* Expression

The greater number of probe sets identified as changed in the 733-siRNA/siRNA-control compared to those of the 337 siRNA treated samples came as a surprise given previous real-time PCR results which showed 733 treated samples as having a greater expression of *Elf5*. However this result also corresponds with the greater reduction in *Elf5* expression compared to the siRNA treated control samples seen in the 733 sample. A real-time PCR measurement for *Elf5* was repeated using cDNA from three of the same samples used for the microarray. However this time the run was performed on the Corbett machine and with a new primer set designed to detect Elf5 (due to the shorter extension time on the Corbett machine). The greater reduction of *Elf5* expression in 733 siRNA treated samples was confirmed. The reason for this apparent contradiction could be because when the real-time PCRs were done prior to

the microarray 12 samples for each treatment was analysed for *Elf5* and *Gapdh* in each run. Due to space limitations on the plate only one treatment was analysed per plate, and the efficiency of each reaction was estimated using four dilutions of one of the samples. This meant the normalised *Elf5* expression calculated for a treatment was compared to the normalised *Elf5* expression for a control measured in a different reaction. This could introduce errors due to differences in the reaction conditions. Another source of error was the use of the dilution series of one sample as an estimator for the efficiency of all samples in a reaction. The efficiency is multiplied to the power of the Ct to give a calculated copy number. Therefore small errors in efficiency can cause a large error in the copy number. These errors could contribute to the incorrect knockdown efficiency calculated. Subsequent to the microarray the expression of *Elf5* was measured using the Rotor gene 6000 (Corbett Life Sciences, Australia). In these runs three samples of each treatment were analysed, enabling the expression of all samples to be compared from a single run. Also the quantitative analysis software application (Corbett life sciences, Australia) allowed an efficiency to be calculated for each reaction. This meant samples with an unusual efficiency were easily detected and not used for expression analysis.

The microarray contained two independent probe sets for Elf5. The fold change is shown in Table 5.1-8 for the two *Elf5* probe sets measured by the microarray, and the relative expression of Elf5 measured by real time rt-PCR. There was a close correlation between the *Elf5* change in expression measured by the Affymetrix microarray and that measured using real-time PCR for all treatment comparisons. For example the average fold change measured for the 337 siRNA was 2.2 fold; using real-time PCR analysis on three samples gave a change of 2.85 fold. The average Elf5 fold changed measured using the microarray for the 733 siRNA treated samples was 3.09, using real time PCR the fold change measured was 4.15.

Table 5.1-8: *Elf5* difference in expression between treatments and their control and the relative *Elf5* expression measured using real time PCR.

	Microarray results for Elf5 probe set 1		Microarray results for Elf5 probe set 2		RT-PCR for *Elf5*		
Contrast	**Fold change**	***P***	**Fold change**	***P***	**Fold change**	**StDev (n=3)**	***P***

337 siRNA vs siRNA control	2.07	1.51E-10	2.34	8.21E-11	2.85	0.05	1.95E-04
733 siRNA vs siRNA control	3.17	3.50E-12	3.01	1.62E-11	4.15	0.03	1.88E-03
TS medium (no growth factors) vs Normal medium	2.57	9.38E-12	3.43	1.40E-12	3.12	0.02	1.05E-02
TS medium + activin (no Fgf4) vs Normal medium	1.94	1.43E-09	2.07	1.38E-09	2.01	0.03	1.78E-02
TS medium + Fgf4 (no activin) vs Normal medium	1.17	1.22E-03	1.36	9.39E-06	1.18	0.07	1.73E-01

Further confidence in the results generated by the microarray experiment was the observation that the probe sets measured to have the greatest change in the *Elf5* siRNA treated were the two probe sets which corresponded to *Elf5*. The top 10 probe sets changed for the 337 and 733 siRNA treated slides are shown in Table 5.1-9 and Table 5.1-10

Table 5.1-9: Top ten probe sets differentially expressed in 773 siRNA traeted samples compared to siRNA control samples [highlighted genes are those changed by both 773 and 337 siRNA treatment.].

GeneName	Product	Fold change
Elf5	E74-like factor 5	-3.17
Elf5	Elf5 probe set 2	-3.01
LCP1/Pls2	lymphocyte cytosolic protein (plastin 2, L)	+2.64
Kap	kidney androgen regulated protein	-2.59
Cyr61	cysteine rich protein 61	-2.54
Cd24a	CD24a antigen	-2.47
LA6	lymphocyte antigen 6 complex	-2.47
Cyr61	cysteine rich protein 61	-2.39
HS3ST3B1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	-2.35

Fxyd5	related to ion channel,	-2.30

Table 5.1-10: The top ten probe sets differentailly expressed in 337 siRNA treated samples compared to siRNA negative control samples [highlighted genes are those changed by both 773 and 337 siRNA treatment.].

GeneName	Product	Fold change
Elf5	Elf5 probe set 2	-2.34
Elf5	E74-like factor 5	-2.07
SYNPO2L	synaptopodin 2 like	-1.76
Hst3st3b1	heparinsulfate (glucosamine) 3-O-sulfotransferase 3B1	-1.76
PPP6C	protein phosphatase 6, catalytic subunit	-1.73
Cyr61	cysteine rich protein 61	-1.71
CXADR	cocksackie virus and adenovirus acceptor	-1.63
Dbt	dihydrolipoamide branched chain transacylase E2	-1.62
PSAT1	phosphoserine aminotransferase	-1.61
Cyr61	cysteine rich protein 61	-1.60
HBXIP	hepatitis B virus x interacting protein	-1.60
Serinc1/Tde1l	tumor differentially expressed 1, like	-1.58

5.1.10 Functional Analysis

Ingenuity Pathway Analysis (IPA v 1.3, 2007) was used to analyse the results to associate biological functional processes, pathways and diseases affected by the treatments, and therefore to associate some overall biological meaning to the results. This was done for each treatment contrast by importing into the software all of the $\log_2$ contrast expression values and their associated *p* value for every probe set. The cut offs described above were also used in this analysis and a *p* value of 0.05 was set. IPA software then displays the functional groups which are over-represented in the set of genes identified as being significantly changed. A *p* value was also calculated by comparing the number of genes identified as changed for a particular functional group compared with the total number of genes belonging to that functional group in the data set of all probes; this depends on the number of genes known/categorised into that functional group. This type of analysis is preferred to grouping genes differentially expressed based on their expression patterns (Allison et al.). The top 20 functional groups identified as significantly over-represented in the set of genes differentially expressed for each treatment are shown in Figure 5.1-16 to Figure 5.1-20.

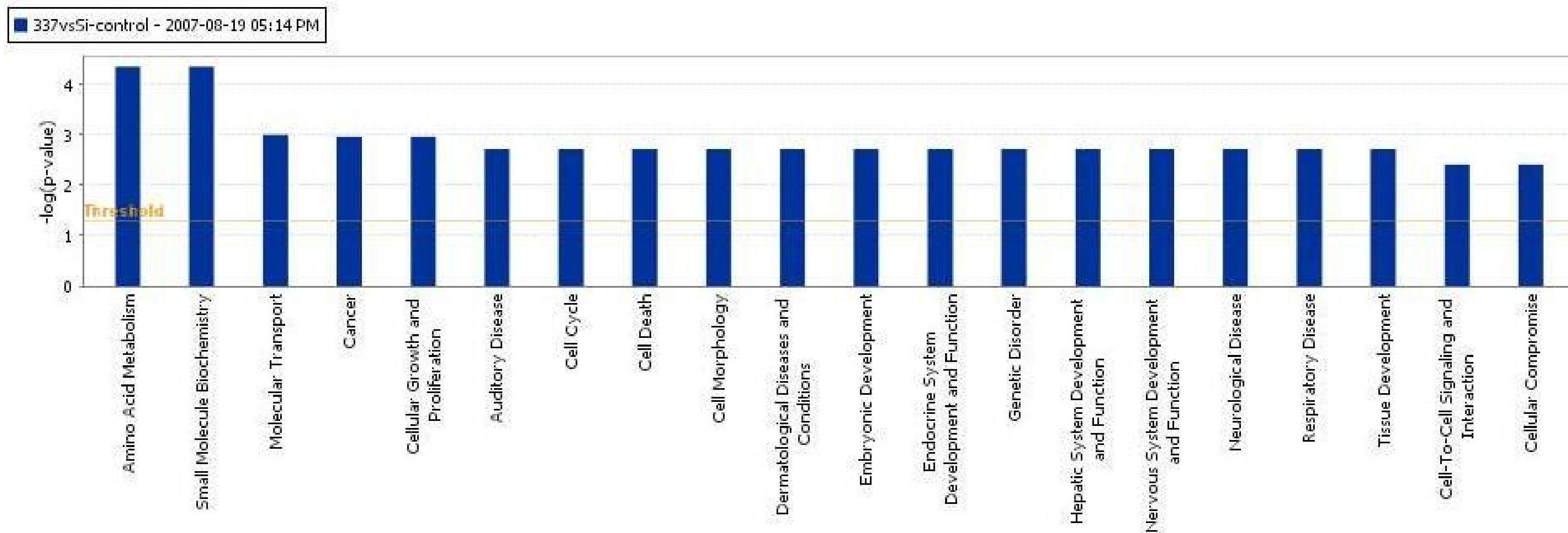


Figure 5.1-16: The top twenty functional categories (ranked by *p value*) which are significantly over-represented in the set of probes differentially expressed (≥1.4 fold change)between 337 siRNA treated mTS cells and siRNA negative control treated mTS cells. Analysis was carried out using IPA 4.0 software. The threshold shown indicates a *p value=0.05*.

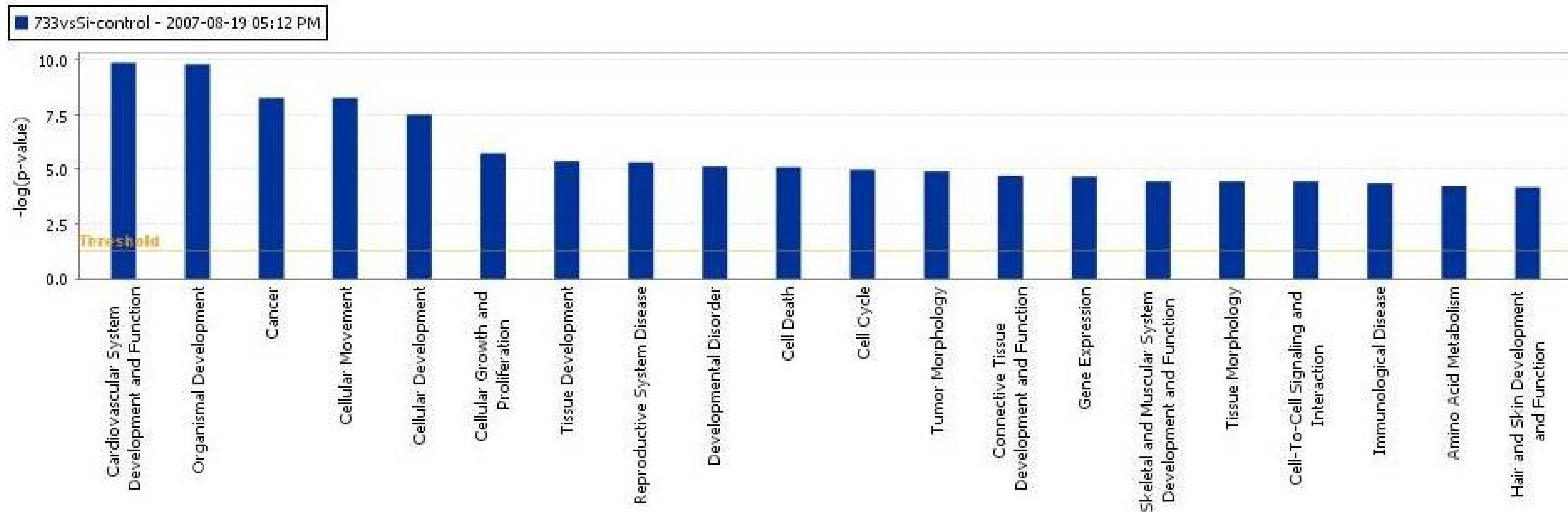


Figure 5.1-17 The top twenty functional categories (ranked by *p value*) which are significantly over-represented in the set of probes differentially expressed (≥1.5 fold change) between 733 siRNA treated mTS cells and siRNA negative control treated mTS cells. Analysis was carried out using IPA 4.0 software, a threshold of *p=0.05* is shown.

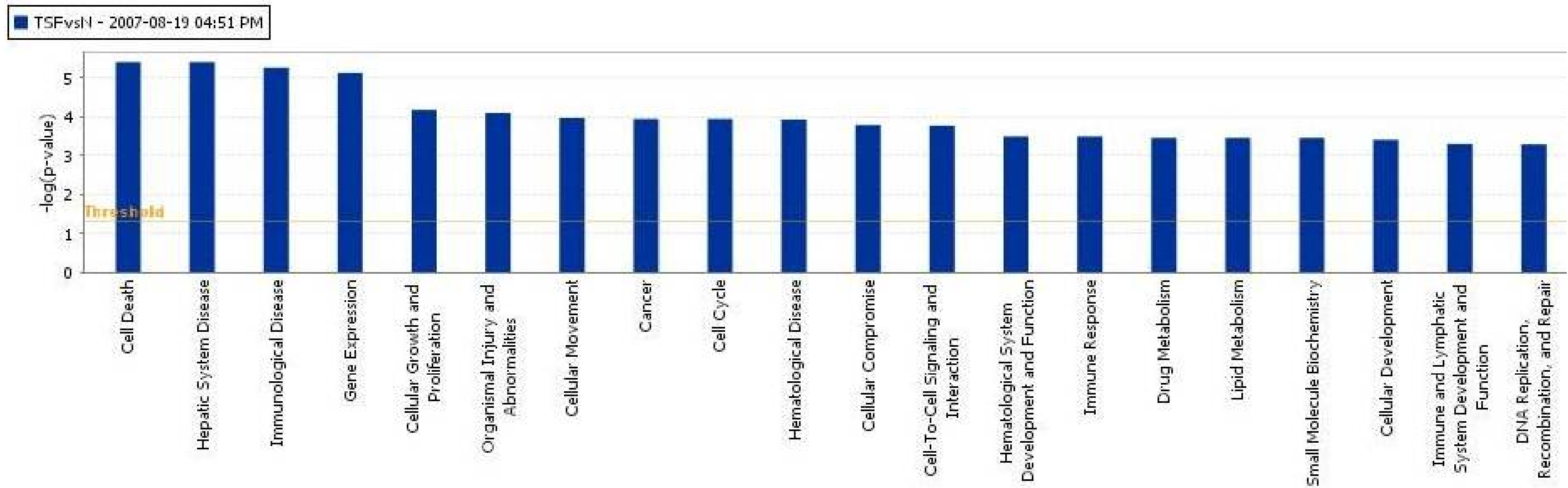


Figure 5.1-18 The top twenty functional categories (ranked by *p value*) which are significantly over-represented in the set of probes differentially expressed (≥2 fold change)between mTS cells grown in TS medium supplemented with Fgf4 growth factors, however without activin, compared to mTS cells grown in normal growth medium containing Fgf4 and activin growth factors. Analysis was carried out using IPA 4.0 software. The threshold shown is for a *p value* of 0.05.

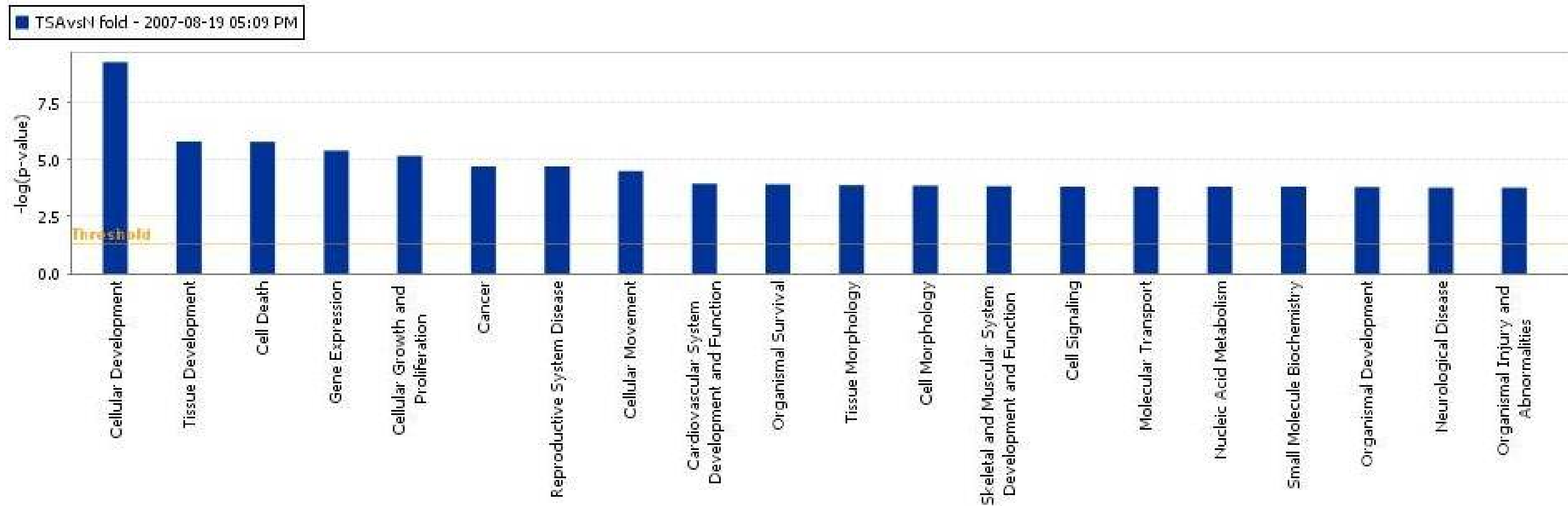


Figure 5.1-19 The top twenty functional categories (ranked by *p value*) which are significantly over-represented in the set of probes differentially expressed between mTS cells grown in TS medium supplemented with avtivin growth factor, however without Fgf4, compared to mTS cells grown in normal growth medium containing Fgf4 and activin growth factors. Analysis was carried out using IPA 4.0 software, the threshold shown indicates a *p value* of 0.05.

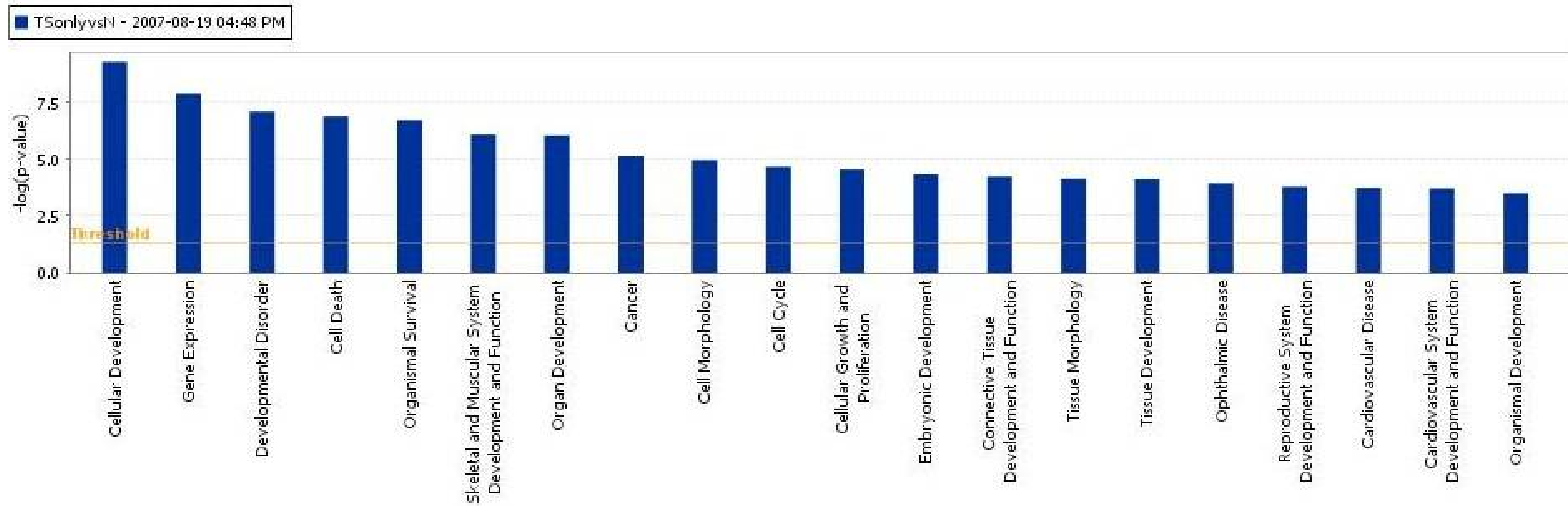


Figure 5.1-20: The top twenty functional categories (ranked by *p value*) which are significantly over-represented in the set of probes differentially expressed between mTS cells grown in TS medium without any Fgf4 or activin supplementation, compared to mTS cells grown in normal growth medium containing Fgf4 and activin growth factors. Analysis was carried out using IPA 4.0 software, the threshold shown indicates a *p value* of 0.05.

5.1.10.1 Functional groups affected by Elf5 knockdown

To see if there was a common effect of knocking down Elf5 using either siRNAs the functional groups identified for each of the *Elf5* siRNA treatments were combined. The total number of functional groups identified as affected by treatment with either siRNA was 70; of these 55 functional groups were found to be significantly over-represented in common between the two siRNA treatments. The functional groups were ordered based on their *p value* and the top 20 groups in common identified in order to establish the major pathways affected by Elf5 knockdown. These are listed in the following table.

Key pathways and biological functions identified as changed due to Elf5 knock down were cancer, cellular growth and proliferation and cell cycle progression. This matches the type of functions expected since *Elf5* is crucial for maintenance of the trophoblast stem cells, so would be expected to be involved in controlling proliferation as well as cell cycle control. Elf5 is also a member of the Ets family of transcription factors (Zhou *et al*., 1998) of which many of its members are associated with cancer. The functional group of embryonic development was also identified as would be expected. Reproductive system disease was also identified. This is significant because malfunctions in the reproductive process as a result of homozygouse knockout of Ets family genes such as *Ets2, Gabpa, Erf* (all involved in placental development) *Pea3*, and *Erm* (involved in male fertility) make the reproductive process the most common target of Ets family transcription factors (Papadaki *et al*., 2007).

Table 5.1-11: The top 20 functional groups (ranked by p value) which show significant expression changes with treatment of both 337 and 733 Elf5 directed siRNAs compared to the control treated siRNA. Functional group analysis was done using IPA 4.0 software v.3

Functional group description	**337 siRNA *p value***	**337 no. of genes**	**733 siRNA *p value***	**733 no. of genes**
Cancer	1.1E-03-4.95E-02	10	5.78E-09-8.34E-03	97
Cellular Growth and Proliferation	1.1E-03-4.21E-02	3	1.99E-06-8.34E-03	90
Cell Cycle	1.95E-03-4.21E-02	5	1.09E-05-7.39E-03	42
Cell Death	1.95E-03-4.93E-02	7	8.34E-06-7.75E-03	77
Amino Acid Metabolism	4.49E-05-4.95E-02	18	6.09E-05-7.67E-03	18

Tissue Development	1.95E-03-4.77E-02	6	4.47E-06-8.34E-03	63
Cell Morphology	1.95E-03-3.83E-02	3	8.18E-05-8.34E-03	43
Small Molecule Biochemistry	4.49E-05-4.95E-02	20	4.05E-04-4.69E-03	14
Connective Tissue Development and Function	3.9E-03-3.64E-02	2	2.08E-05-6.22E-03	31
Cell-To-Cell Signaling and Interaction	3.9E-03-4.95E-02	4	3.71E-05-7.34E-03	53
Embryonic Development	1.95E-03-4.77E-02	5	2.25E-04-8.3E-03	31
Gene Expression	3.9E-03-2.89E-02	3	2.22E-05-6.08E-03	62
Reproductive System Disease	3.9E-03-4.95E-02	8	4.99E-06-6.55E-03	50
Cardiovascular System Development and Function	7.78E-03-4.95E-02	4	1.42E-10-8.34E-03	41
Respiratory Disease	1.95E-03-3.27E-02	2	2.27E-04-5.53E-03	13
Endocrine System Development and Function	1.95E-03-3.46E-02	3	4.47E-04-4.69E-03	7
Tumor Morphology	3.9E-03-1.55E-02	1	1.28E-05-7.96E-03	27
Skeletal and Muscular System Development and Function	3.9E-03-3.64E-02	2	3.69E-05-7.42E-03	24
Molecular Transport	1.01E-03-4.77E-02	8	2.04E-03-2.04E-03	2
Tissue Morphology	3.9E-03-4.95E-02	3	3.69E-05-8.3E-03	48

5.1.11 Pathway analysis

The probe sets identified as significantly changed in the 733 and 337 siRNA treated samples compared to the siRNA negative control samples were used to perform a pathway analysis(with IPA software), to gain an understanding of the known molecular

interactions which occur in this set of genes. The IPA software uses published interactions of protein or protein/nucleotide sequence to create a network of the known interactions. The network produced is shown in Figure 5.1-21. Key genes indicated to be important in the pathway however not identified in the microarray were vascular endothelial growth factor C (VEGF-C), Errβ, TGFβ, and MYC.

Network 1 : 337u773vsi-expressiopn - 2007-08-25 01:00 PM : 337u773vsi-expressiopn.xls

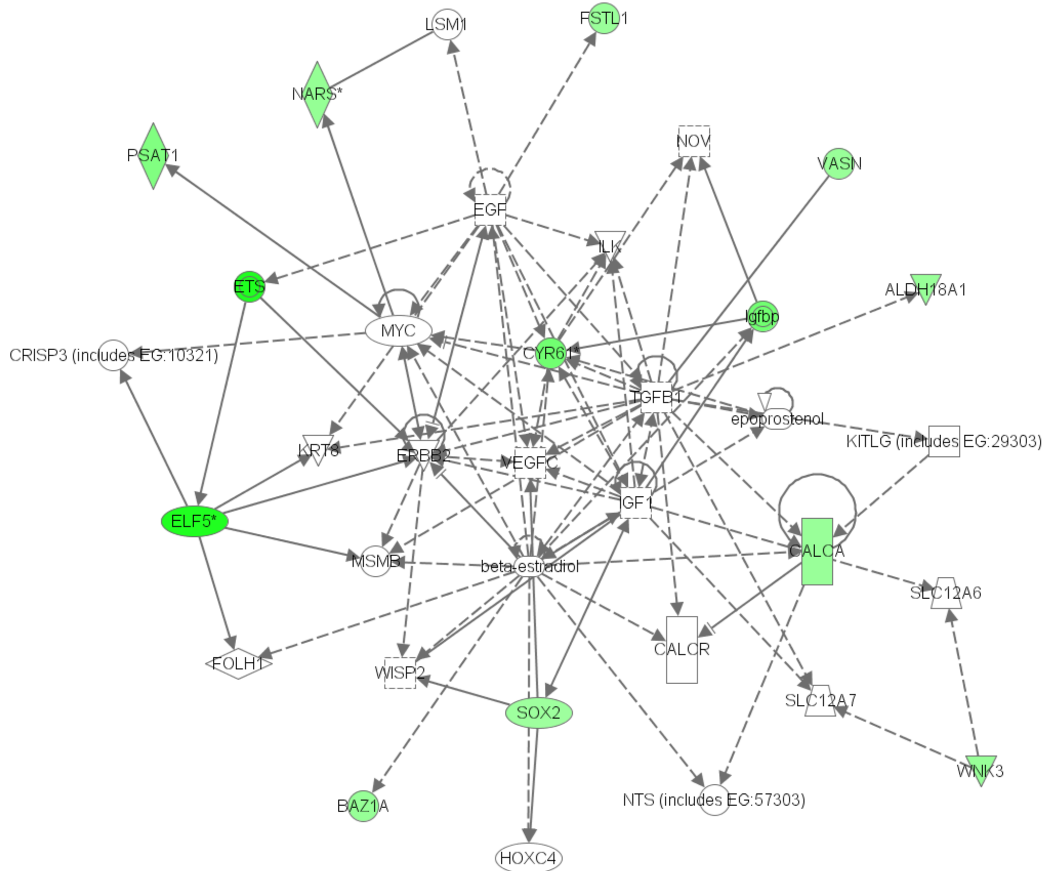


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Figure 5.1-21: Pathway analysis using IPA software to show the known interactions between genes identified as significantly affected by 337 and 733 Elf5 siRNA oligonucleotides compared to control siRNA treatment.

5.1.12 Intersections of Treatments

To lower the false discovery rate (the number of probe sets identified as differentially expressed when they are not) and to find probe sets which were affected by more than one treatment, the list of unique probes which had made the cut-offs were filtered for intersections. The Venn diagram, which shows the number of probe sets affected by more than one treatment, is shown below (Figure 5.1-22)

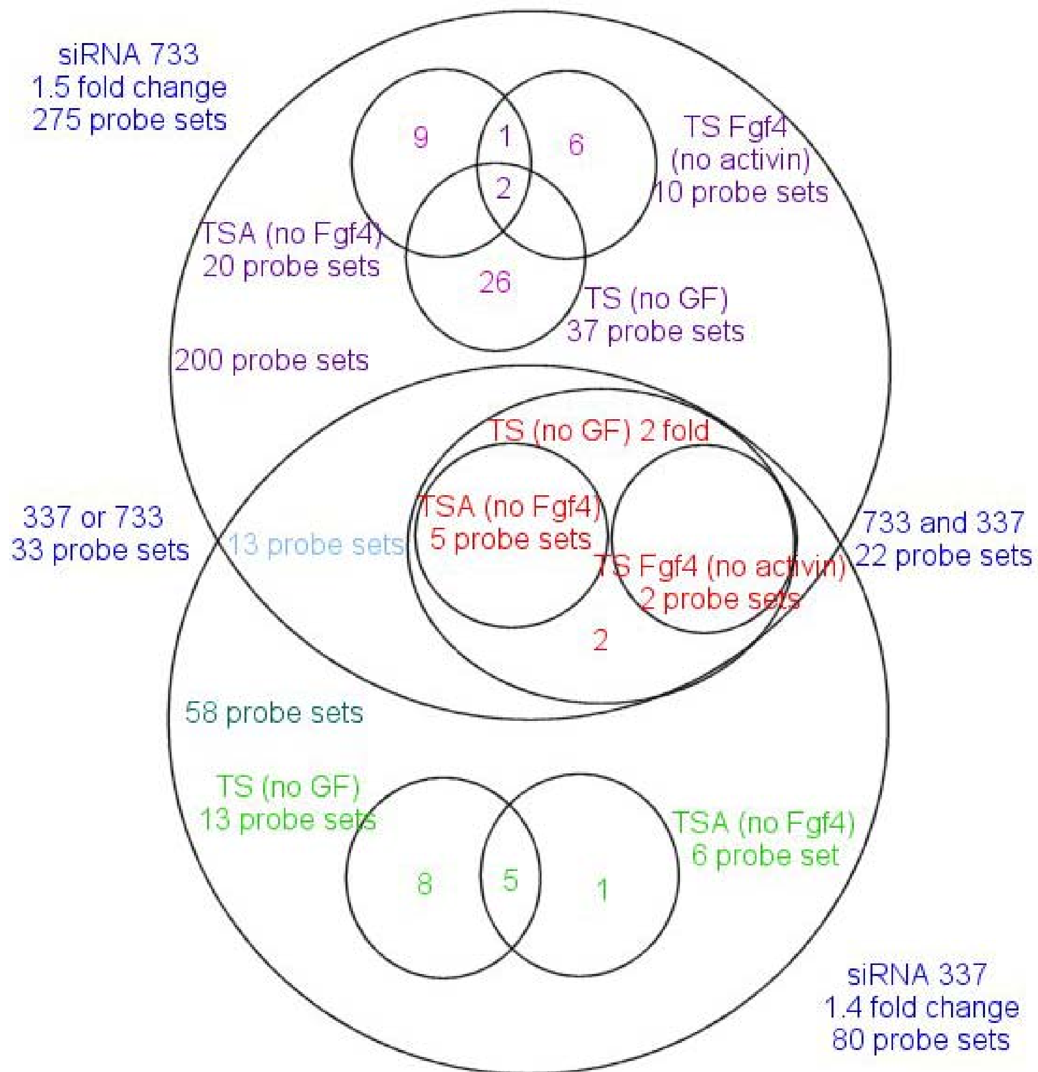

Figure 5.1-22: Venn diagram showing the number of probe sets differentially expressed with a common treatment.

A siRNA oligo may produce off target effects (Bridge *et al.*, 2003) therefore the set of genes of greatest interest are those which are differentially expressed when both siRNA oligos are used. A set of 22 probe sets were identified as having changed when either *Elf5* siRNA oligo was used. This set was then further analysed to see if any of these probe sets are also altered when the growth factors activin/Fgf4 were removed, to indicate genes which may be involved in both growth factor maintenance of mTS cells and regulated by Elf5. A subset of nine probesets was found (indicated in red in the Venn diagram). The other overlapping treatments investigated were the intersections

of either *Elf5* siRNA oligos with the growth factor experiments. There were 53 probe sets in common between 733 siRNA treatment (1.5 fold) and removal of activin/Fgf4. Only 14 probe sets were changed by growth factor removal (2 fold) and 337 siRNA treatment. A significant group of probe sets was unique to each oligo. The biggest reason for this is the 733 oligo had a greater effect on Elf5 expression, therefore it would be expected that a greater number of down stream genes would also be significantly changed; however with 337 treatment the change in Elf5 could have not been great enough for these genes to appear significantly changed. The other reason for the different sets of genes identified could be non-specific effects due to the particular sequence of each of the oligos, causing RNA interference effects on genes other than Elf5.

5.1.12.1 Annotation of Genes of Interest

The probe sets from the intersection of:

i. 337 and 733 and any of the growth factor removal treatments (9 probesets)

ii. 337 and 733, but not any of the growth factor treatments (13 probe sets)

iii. 337 and any of the growth factor treatments, but not 733 (14 probe sets)

iv. 733 and any of the growth factor treatments but not 337 (53 probe sets)

v. The set of genes which changed by greater than 3.5 fold in any of the growth factor experiments (57 probe sets)

were annotated for gene name, description and an outline of the function of that gene using IPA 4.0 software (Ingenuity pathway analysis, v3, 2006). Many of the probe sets which, using the Affymetrix annotation had not been previously associated with a gene name, were found to be for the same gene. For example in the set of "unique" probe sets for the 337/733/GF all union, Cyr61 was found to occur twice. The annotated table of genes showing the affymetrix probe number, matching gene name, description as well as the fold change and the *p value* are given in Table 5.1-12.

Several genes known to be involved in placental development were identified as significantly changed. In the set of probes of key interest (the union of

337/733/growth factor treatments) the genes matched were: *Cyr61* (twice), *Sox2*, *Elf5* (twice), *Hst3st3B1*, *Wdr40b*, *Synopl* and a probe set not matched by ingenuity. The fact Elf5 was identified in this union gives some confidence to the results of the microarray. The other union of key interest is that between 337 and 733 siRNA treatments. Of the 22 genes in this group was Follistatin which is known to be expressed in placental tissue and to be important in controlling activin/Tgfβsignalling (Jones *et al*., 2002). Tgfβ signalling is one of the pathways essential for maintenance of mTS cells (Erlebacher et al., 2004). Vasorin was also identified in this set of genes and is known to be involved in the Tgfβ pathway as well (Ikeda *et al*., 2004). Calca (calcitonin) has been implicated to play an important role in calcium transport and signalling in placental and early embryonic development (Kovacs *et al*., 2002; Wang *et al*., 1998).

In the set of genes annotated from removal of Fgf4 and activin growth factors were a number of genes that are well known to be expressed in mTS cells and their differentiated subtypes. These genes included *Bmp4*, *Ascl2/Mash2, Mmp9*, *Eomes* and *Errβ*. *Eomes* and *Errβ* are genes downstream of Fgf4 signalling and are required for mTS cell proliferation (Cross *et al*., 2003), these genes were both down regulated when Fgf4 signalling was removed. *Mash2* is expressed in the mTS down stream spongiotrophoblast cell type (Cross et al., 2003) and was upregulated, consistent with the mTS cells undergoing differentiation.

Many of the genes found to be differentially expressed were ones which are known to play a role in placental development or mTS cell maintenance and differentiation. These genes also followed the expected pattern such as up or down regulation with differentiation. These results indicate that the microarray results do have biological meaning and the genes differentially expressed are valid.

Table 5.1-12: The complete set of probes of interest (affymtrix probe number, matched to a gene using IPA 4.0 software v 3) identified after fold-change cutoffs had been applied and probe sets classified based on common to unions of treatments. The probe sets for each union are colour coded. Red= the union of siRNAs 337 and 773 and by all growth factor treatments(1.4 fold, 1.5 fold and 2 fold respectively; i.e. the set of genes which are affected by both Elf5 knockdown and growth factor removal); blue=union of 337 and 733 but not identified as significantly changed by growth factor removal (1.4 fold and 1.5 fold respectively; i.e. Elf5 regulated only)green=probe sets affected by 337 siRNA treatment and growth factor removal, however not 733 treatment (1.4 fold and 2 fold respectively; i.e. specific effects of the 337 siRNA oligo); purple=probe sets changed by both 733 siRNA oligo treatment and growth factor removal but not 337 siRNA treatment(1.5 fold and 2 fold respectively; i.e. specific to the 733 siRNA oligo); grey=probe sets not changed by elf5 siRNA treatment however affected by a 3.5-fold or greater with removal of growth factors (activin, Fgf4 or both), i.e. genes which are Elf5 independent. . Shaded boxes are those values which met the fold change cutoffs.

matched term	name	description	TSA vs N	TSF vs N	TS vs N	337 vs Si-	773 vs Si-	N vs Si-	Pvalue TSA vs N	Pvalue TSF vs N	Pvalue TS vs N	Pvalue 337 vs Si-	Pvalue 773 vs Si-	Pvalue N vs Si-
1416039_x_at, 1438133_a_at	CYR61	cysteine-rich, angiogenic inducer, 61	-1.30	-1.56	-2.02	-1.60	-2.39	-1.21	1.33E-02	3.81E-04	2.48E-06	1.00E-04	8.78E-07	3.71E-02
1438133_a_at	cyr61	cysteine-rich, angiogenic inducer, 62	-1.31	-1.57	-2.18	-1.71	-2.54	-1.14	6.76E-03	1.77E-04	3.77E-07	1.34E-05	1.86E-07	1.07E-01
1416967_at	SOX2	SRY (sex determining region Y)-box 2	-2.33	-1.07	-8.62	-1.44	-1.88	1.00	1.03E-06	4.70E-01	1.71E-11	6.54E-04	1.67E-05	9.87E-01
1419555_at, 1419556_at	ELF5	E74-like factor 5 (ets domain transcription factor)	-1.94	-1.17	-2.57	-2.07	-3.17	-1.08	1.43E-09	1.22E-03	9.38E-12	1.51E-10	3.50E-12	3.78E-02

1419556_at	Elf5	E74-like factor 5 (ets domain transcription factor)	-2.07	-1.36	-3.43	-2.34	-3.01	1.03	1.38E-09	9.39E-06	1.40E-12	8.21E-11	1.62E-11	3.73E-01
1433977_at	HS3ST3B1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	-2.29	-1.00	-5.17	-1.76	-2.35	1.22	8.33E-07	9.73E-01	2.04E-10	1.15E-05	6.10E-07	2.43E-02
1434170_at	WDR40B	WD repeat domain 40B	-3.79	-1.30	-3.32	-1.43	-1.58	1.31	5.80E-09	9.18E-03	5.36E-09	5.69E-04	1.84E-04	4.30E-03
1447657_s_at	SYNPO2L	synaptopodin 2-like	-1.34	-2.22	-4.17	-1.76	-2.01	1.20	2.10E-03	2.86E-07	1.91E-10	2.75E-06	1.05E-06	1.71E-02
1455961_at	No term matched via ingenuity		1.11	-2.57	-2.37	-1.48	-2.59	-1.14	2.42E-01	2.36E-07	1.93E-07	3.12E-04	2.21E-07	1.21E-01
1437325_x_at	ALDH18A1	aldehyde dehydrogenase 18 family, member A1	-1.60	1.04	1.63	-1.48	-1.61	-1.46	8.23E-04	7.42E-01	2.62E-04	1.44E-03	7.25E-04	1.66E-03
1444038_at	AU015836	expressed sequence AU015836	-1.96	1.00	1.95	-1.47	-2.27	-1.44	8.31E-05	3.96E-02	2.05E-05	9.51E-06	2.14E-06	6.94E-05
1433599_at	BAZ1A	bromodomain adjacent to zinc finger domain, 1A	-1.17	-1.09	1.02	-1.48	-1.74	-1.20	1.90E-02	1.66E-01	7.40E-01	8.61E-06	9.68E-07	3.93E-03

1452004_at	CALCA	calcitonin/calcitonin-related polypeptide, alpha	-1.95	-1.60	-1.96	-1.47	-1.63	1.01	3.61E-06	9.41E-05	1.13E-06	1.90E-04	6.71E-05	8.36E-01
1452828_at	FBXO21	F-box protein 21	-1.02	-1.08	1.52	-1.48	-1.70	-1.37	6.89E-01	4.84E-02	5.37E-08	1.10E-07	1.53E-08	9.21E-07
1437200_at	FCHO2	FCH domain only 2	1.27	1.06	1.17	-1.50	-1.65	-1.31	1.64E-01	7.06E-01	3.00E-01	1.75E-02	1.01E-02	8.53E-02
1416221_at	FSTL1	follistatin-like 1	-1.41	-1.20	-1.12	-1.47	-1.74	-1.06	4.34E-05	4.97E-03	3.84E-02	5.57E-06	4.54E-07	2.25E-01
1452866_at	NARS	asparaginyl-tRNA synthetase	-1.39	1.04	1.45	-1.49	-1.74	-1.48	5.08E-05	4.66E-01	6.37E-06	3.16E-06	3.81E-07	3.79E-06
1428666_at, 1452866_at	NARS	asparaginyl-tRNA synthetase	-1.50	-1.00	1.16	-1.49	-1.68	-1.40	3.54E-05	9.43E-01	2.09E-02	1.50E-05	3.46E-06	6.75E-05
1454607_s_at	PSAT1	phosphoserine aminotransferase 1	-1.63	1.10	1.29	-1.61	-1.55	-1.25	4.30E-03	5.09E-01	5.99E-02	2.52E-03	8.13E-03	9.96E-02
1424556_at	PYCR1	pyrroline-5-carboxylate reductase 1	-1.41	-1.14	1.43	-1.47	-1.66	-1.37	3.49E-06	9.68E-01	1.29E-06	2.11E-04	5.12E-07	3.26E-04
1455812_x_at	VASN	vasorin	1.33	1.06	1.36	-1.47	-1.57	-1.19	1.13E-02	5.26E-01	4.25E-03	8.29E-04	6.07E-04	6.02E-02
1443924_at	WNK3	WNK lysine deficient protein kinase 3	1.05	-1.23	-1.27	-1.48	-1.87	-1.21	3.73E-01	3.29E-03	4.80E-04	6.37E-06	1.86E-07	2.66E-03
1416530_a_at	NP	nucleoside phosphorylase	-1.29	-1.12	-2.75	-1.42	-1.25	-1.40	1.18E-04	2.13E-02	3.04E-11	2.13E-06	2.87E-04	3.10E-06

1418152_at	NSBP1	nucleosomal binding protein 1	-2.02	1.18	1.18	-1.42	-1.03	-1.08	1.09E-05	1.03E-01	7.64E-02	1.40E-03	7.80E-01	3.89E-01
1418681_at	ALG13	asparagine-linked glycosylation 13 homolog (S. cerevisiae)	-1.33	-1.39	-2.64	-1.48	-1.46	1.20	3.06E-03	1.12E-03	1.77E-08	1.22E-04	3.78E-04	1.92E-02
1423271_at	GJB2/connexion26	gap junction protein, beta 2, 26kDa	1.22	1.08	3.26	1.45	-1.10	-1.04	4.03E-03	2.05E-01	6.62E-11	1.05E-05	9.61E-02	4.62E-01
1427355_at	CALCA	calcitonin/calcitonin-related polypeptide, alpha	-2.36	-1.77	-2.65	-1.52	-1.43	1.19	1.83E-07	1.05E-05	1.54E-08	6.30E-05	5.67E-04	2.31E-02
1436905_x_at	LAPTM5	lysosomal associated multispanning membrane protein 5	-1.18	-1.44	-2.41	-1.57	-1.40	1.21	3.83E-02	2.45E-04	1.85E-08	1.46E-05	4.63E-04	1.04E-02
1438750_at	ATRX	alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)	-1.38	-1.03	-3.20	-1.50	-1.05	1.29	1.74E-02	8.12E-01	2.42E-07	2.58E-03	6.65E-01	3.07E-02

1440910_at	ESTs	No match found in IPA	-1.66	1.13	-4.02	-1.55	-1.21	1.19	1.51E-05	1.02E-01	1.46E-10	2.05E-05	1.79E-02	1.53E-02
1442073_at	ESTs	No match found in IPA	-2.64	1.05	-5.68	-1.44	-1.14	1.17	7.50E-05	7.68E-01	9.47E-08	2.65E-02	4.14E-01	2.93E-01
1437059_at	SOX21	SRY (sex determining region Y)-box 21	-2.49	1.02	-3.23	-1.50	-1.34	1.27	5.81E-07	8.20E-01	1.39E-08	3.58E-04	6.81E-03	1.17E-02
1451835_at	SOX21	SRY (sex determining region Y)-box 22	-2.10	1.12	-2.30	-1.46	-1.41	1.07	7.00E-08	2.26E-04	6.59E-09	9.61E-06	8.73E-04	8.47E-04
1455195_at	RPS24	ribosomal protein S24	-2.01	-1.35	-2.19	-1.46	-1.29	1.25	8.89E-04	5.36E-01	2.62E-07	9.54E-04	1.25E-03	1.67E-01
1455425_at	BB001228	expressed sequence BB001228	-1.50	-1.06	-2.42	-1.43	-1.47	1.13	1.09E-02	9.38E-01	2.83E-06	7.08E-04	2.93E-03	3.11E-01
1456036_x_at	GSTO1	glutathione S-transferase omega 1	-1.36	-1.01	-2.17	-1.51	-1.46	1.10	8.14E-06	2.75E-01	8.96E-07	9.11E-04	4.11E-03	4.32E-01

1416630_at	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	-1.18	2.20	2.01	-1.06	2.20	-1.32	1.49E-03	4.09E-10	4.41E-10	1.16E-01	4.19E-10	5.75E-06
1417185_at	LY6A	lymphocyte antigen 6 complex, locus A	-1.51	-1.80	-5.25	-1.01	2.47	-1.14	3.46E-05	1.13E-06	6.04E-12	8.59E-01	1.37E-08	3.14E-02
1417328_at	ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1	-1.49	-1.04	-2.04	-1.10	-1.66	-1.36	1.13E-05	4.38E-01	1.03E-08	6.88E-02	1.08E-06	4.07E-05
1417426_at	PRG1 (includes EG:5552)	proteoglycan 1, secretory granule	-2.11	-1.55	-7.01	-1.19	2.26	-1.33	1.49E-05	1.28E-03	2.67E-10	8.65E-02	6.71E-06	1.04E-02
no match found in ingenuity	RIKEN cDNA 1110070A02		-1.12	-1.32	-2.39	-1.03	-1.68	-1.13	2.26E-01	7.59E-03	2.09E-07	6.87E-01	8.54E-05	1.34E-01
1417487_at	FOSL1	FOS-like antigen 1	-2.02	-1.24	-2.26	-1.17	-1.68	-1.46	1.60E-06	1.71E-02	1.19E-07	4.06E-02	2.90E-05	1.76E-04

1418296_at	FXYD5	FXYD domain containing ion transport regulator 5	-1.45	-1.12	-2.13	-1.08	2.30	-1.34	2.28E-04	1.19E-01	9.99E-08	2.59E-01	1.16E-07	6.01E-04
1418762_at	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	-1.60	-1.55	-4.72	-1.09	-1.61	-1.02	5.79E-05	1.08E-04	1.04E-10	2.35E-01	4.92E-05	7.73E-01
1443906_at, 1460242_at	CD55		-1.61	-1.61	-4.10	-1.23	-1.61	-1.11	6.63E-04	7.03E-04	8.21E-09	4.12E-02	6.51E-04	2.89E-01
	CD55		-1.59	-1.89	-6.26	-1.05	-1.53	-1.12	1.00E-04	5.79E-06	2.89E-11	5.26E-01	2.12E-04	1.42E-01
1418805_at	SCT	secretin	2.21	2.17	2.16	-1.30	-1.83	-1.19	2.03E-09	2.54E-09	8.62E-10	4.61E-05	3.43E-08	1.03E-03
1420498_a_at	DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	2.55	-2.42	-1.01	-1.07	-1.88	-1.18	6.39E-11	1.17E-10	8.07E-01	6.66E-02	4.45E-09	4.65E-04
1420911_a_at	MFGE8	milk fat globule-EGF factor 8 protein	-1.00	-1.86	-2.07	-1.08	-1.56	-1.03	9.67E-01	9.26E-08	5.72E-09	1.30E-01	2.61E-06	4.97E-01

1421882_a_at	ELAVL2	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	-1.59	-1.61	-2.10	-1.12	-1.97	-1.11	1.23E-04	9.82E-05	5.12E-07	1.33E-01	3.72E-06	1.60E-01
1422776_at	SERPINB8	serpin peptidase inhibitor, clade B (ovalbumin), member 8	-1.22	-1.83	-2.06	-1.36	-1.88	-1.12	3.45E-02	1.50E-05	9.16E-07	1.58E-03	1.02E-05	1.47E-01
1422851_at	HMGA2	high mobility group AT-hook 2	-2.15	-1.46	-1.62	-1.26	-2.18	-1.11	1.59E-06	7.72E-04	4.26E-05	9.38E-03	1.34E-06	1.72E-01
1450780_s_at, 1450781_at			-2.10	-1.39	-1.49	-1.18	-1.93	-1.08	4.03E-06	3.29E-03	3.61E-04	6.00E-02	1.24E-05	3.52E-01
			-2.19	-1.60	-1.86	-1.22	-1.80	-1.01	1.13E-07	1.67E-05	3.99E-07	5.48E-03	2.08E-06	8.93E-01
1423424_at	ZIC3	Zic family member 3 heterotaxy 1 (odd-paired homolog, Drosophila)	-3.51	-1.51	-7.47	-1.05	-2.29	-1.08	8.59E-07	7.85E-03	2.03E-09	6.78E-01	4.39E-05	5.27E-01
1438737_at	ZIC4		-2.05	-1.25	-2.39	-1.22	-1.62	-1.04	7.35E-06	3.22E-02	3.51E-07	3.31E-02	2.45E-04	6.12E-01

1423747_a_at	PDK1	pyruvate dehydrogenase kinase, isozyme 1	-2.03	-1.04	-1.12	-1.11	-2.22	-1.03	1.35E-03	8.39E-01	4.68E-01	4.87E-01	5.67E-04	8.66E-01
1423952_a_at	KRT7	keratin 7	2.08	-1.52	-1.78	-1.04	-1.87	-1.14	2.98E-08	7.95E-06	1.10E-07	4.29E-01	1.55E-07	2.16E-02
1424938_at	STEAP1	six transmembrane epithelial antigen of the prostate 1	-1.17	-2.26	-1.65	-1.32	-1.57	-1.06	2.60E-02	3.73E-08	1.79E-06	3.27E-04	1.34E-05	3.39E-01
1425811_a_at	CSRP1	cysteine and glycine-rich protein 1	2.00	-1.39	-1.96	-1.00	-1.78	-1.06	4.26E-07	3.99E-04	1.96E-07	9.91E-01	2.79E-06	3.28E-01
1426243_at	CTH	cystathionase (cystathionine gamma-lyase)	-1.17	-1.70	-2.82	-1.32	-1.55	-1.00	8.91E-03	4.60E-07	1.22E-10	7.49E-05	3.20E-06	9.55E-01
1426258_at	SORL1 (includes EG:6653)	sortilin-related receptor, L(DLR class) A repeats-containing	-2.27	-1.46	-3.34	-1.02	-1.54	-1.09	9.68E-07	9.16E-04	5.81E-09	7.92E-01	3.32E-04	2.59E-01
1427364_a_at	ODC1	ornithine decarboxylase 1	-1.58	-1.14	-2.05	-1.01	-1.84	-1.42	4.44E-05	8.92E-02	1.82E-07	9.24E-01	2.86E-06	1.68E-04
1428187_at	CD47	CD47 molecule	2.20	-1.34	-1.24	-1.09	-1.75	-1.30	7.30E-08	6.32E-04	2.45E-03	1.50E-01	2.21E-06	7.22E-04

1449507_a_at	CD47		2.01	-1.45	-1.29	-1.12	-1.80	-1.24	2.35E-08	1.17E-05	1.43E-04	2.45E-02	1.34E-07	5.33E-04
1428209_at	BEXL1	brain expressed X-linked-like 1	-1.90	-2.11	-1.33	-1.36	-2.01	-1.06	1.01E-08	2.11E-09	1.12E-05	5.84E-06	4.43E-09	1.40E-01
1428622_at	DEPDC6	DEP domain containing 6	-1.34	-1.50	-2.26	-1.16	-1.78	-1.13	1.40E-04	8.34E-06	1.98E-09	8.18E-03	2.48E-07	2.54E-02
1443579_s_at, 1451348_at	DEPDC6		-1.49	-1.60	-2.67	-1.09	-1.73	-1.00	1.05E-05	2.01E-06	3.15E-10	9.18E-02	4.53E-07	9.85E-01
	DEPDC6		-1.35	-1.62	-2.59	-1.18	-1.86	-1.04	3.59E-03	9.65E-05	4.46E-08	4.66E-02	9.63E-06	5.88E-01
1428662_a_at	HOP	homeodomain -only protein	-1.35	-1.21	-2.22	-1.13	-1.78	-1.13	3.18E-04	6.51E-03	8.67E-09	3.76E-02	7.24E-07	3.38E-02
1451776_s_at	HOP	homeodomain -only protein	-1.28	-1.23	-2.04	-1.07	-1.64	-1.18	1.70E-04	6.05E-04	1.63E-09	1.21E-01	2.37E-07	1.53E-03
1429646_at	1700112C13RIK	RIKEN cDNA 1700112C13 gene	-2.38	-2.16	-3.03	-1.23	-2.19	-1.05	1.67E-08	5.81E-08	3.72E-10	2.63E-03	4.88E-08	3.63E-01
1429759_at	RPS6KA6	ribosomal protein S6 kinase, 90kDa, polypeptide 6	-1.13	-1.11	2.27	-1.08	-2.16	-1.27	1.18E-01	1.78E-01	8.45E-08	2.80E-01	5.04E-07	4.18E-03
1429761_at	RTN1	reticulon 1	-1.61	-1.18	2.68	-1.30	-1.55	-1.29	4.20E-04	1.15E-01	1.89E-07	1.15E-02	8.07E-04	1.35E-02
1434025_at	no match found in ingenuity	**Klf5**	-1.60	-1.30	-2.80	-1.11	-1.84	-1.14	2.05E-04	1.08E-02	3.73E-08	2.04E-01	2.07E-05	1.18E-01

1437277_x_at	TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	-1.93	2.04	-1.92	-1.08	-1.60	-1.01	1.78E-07	7.91E-08	6.18E-08	1.65E-01	5.06E-06	8.00E-01
1438118_x_at	VIM	vimentin	-1.62	-2.15	-1.97	-1.05	-1.53	-1.28	4.65E-05	5.89E-07	6.45E-07	5.18E-01	1.49E-04	3.48E-03
1439341_at	AK220484	cDNA sequence AK220484	2.20	-1.56	-1.37	-1.13	-1.69	-1.31	7.11E-10	2.71E-07	3.00E-06	5.97E-03	5.51E-08	1.33E-05
1447640_s_at	PBX3	pre-B-cell leukemia transcription factor 3	-3.39	-1.39	-4.13	-1.10	-1.64	-1.07	3.31E-09	8.04E-04	2.04E-10	1.59E-01	2.98E-05	3.43E-01
1448562_at	UPP1	uridine phosphorylase 1	-1.49	-2.69	-1.46	-1.06	-4.98	-1.39	7.37E-04	1.78E-07	4.80E-04	4.60E-01	1.11E-09	1.33E-03
1448752_at	CA2	carbonic anhydrase II	-1.86	-1.56	-2.39	-1.02	-1.57	-1.06	5.68E-09	1.72E-07	4.31E-11	6.72E-01	1.55E-07	1.19E-01
1448804_at	CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	-1.59	-1.26	-2.19	-1.16	2.13	-1.14	3.30E-06	1.24E-03	5.13E-09	9.96E-03	2.41E-08	1.95E-02

1449235_at	FASLG	Fas ligand (TNF superfamily, member 6)	-1.13	2.16	-1.16	-1.06	-1.79	-1.21	2.89E-02	7.56E-09	6.86E-03	2.11E-01	1.34E-07	1.31E-03
1449852_a_at	EHD4	EH-domain containing 4	-1.82	-1.42	-2.55	-1.06	-1.88	-1.29	7.40E-06	7.83E-04	2.74E-08	4.46E-01	4.58E-06	3.44E-03
1450791_at	NPPB	natriuretic peptide precursor B	-1.73	-1.57	-2.89	-1.40	2.38	-1.49	8.49E-06	5.06E-05	3.27E-09	2.50E-04	8.68E-08	5.91E-05
1453304_s_at	LY6E	lymphocyte antigen 6 complex, locus E	-1.62	-1.03	-2.30	-1.06	-1.76	-1.27	1.47E-05	6.52E-01	1.94E-08	3.10E-01	3.18E-06	1.89E-03
1454869_at	WDR40B	WD repeat domain 40B	-3.13	-1.20	-3.65	-1.39	-1.73	-1.42	1.15E-05	2.47E-01	1.18E-06	3.35E-02	4.16E-03	2.45E-02
1456326_at	GM784	gene model 784, (NCBI)	-4.50	-1.91	-3.28	-1.33	-1.98	-1.24	9.00E-11	6.18E-07	3.43E-10	4.19E-04	3.80E-07	2.95E-03
1458667_at	KIAA0980	KIAA0980 protein	-1.91	-1.11	-2.16	-1.31	-1.62	-1.02	4.74E-03	5.67E-01	6.50E-04	1.31E-01	2.41E-02	8.94E-01
1430516_at	**4930428B01Rik**	RIKEN cDNA 4930428B01 gene	-2.80	-1.09	-3.52	-1.08	-1.00	-1.23	1.56E-08	2.40E-01	5.71E-10	2.58E-01	9.97E-01	8.38E-03
1434528_at	**AARD**	alanine and arginine rich doamin containing protein	-2.00	-1.07	-5.62	-1.07	-1.13	-1.07	7.84E-09	1.37E-01	1.24E-13	1.26E-01	2.01E-02	1.12E-01

1417976_at	**Ada**	adenosine deaminase	-1.57	-1.43	-3.73	-1.38	-1.10	-1.32	1.70E-05	1.37E-04	8.77E-11	1.21E-04	1.64E-01	3.80E-04
1454822_x_at	**Apcdd1/EIG180**	adenomatosis polyposis coli downregulated/ethanol induced gene product EIG180	-2.12	-1.41	-3.77	-1.09	-1.11	-1.29	3.07E-06	2.25E-03	2.83E-09	2.71E-01	2.66E-01	7.06E-03
1422396_s_at	**Ascl2**	mammalian achaete scute homolog 2	-1.57	-1.16	4.17	-1.13	-1.04	-1.09	1.20E-04	8.36E-02	3.83E-10	1.00E-01	6.23E-01	2.27E-01
1432018_at	**Ascl2/Mash2**	achaete-scute complex homolog 2 (Drosophilia)	-1.90	-1.40	5.27	-1.24	-1.23	-1.23	1.42E-04	1.29E-02	4.38E-09	5.87E-02	9.82E-02	6.26E-02
1460514_s_at	**Ascl2/Mash2**	achaete-scute complex homolog 2 (Drosophilia)	-1.68	-1.30	4.91	-1.25	-1.17	-1.24	2.78E-05	5.58E-03	9.94E-11	7.99E-03	5.84E-02	9.69E-03

1420947_at	**ATRX**	apha thalassemia/mental retardation syndrom X linked, X-linked nuclear protein	-1.44	-1.18	-3.70	-1.03	-1.18	-1.06	2.10E-02	2.57E-01	3.30E-07	8.27E-01	2.48E-01	6.22E-01
1440156_s_at	**AV026525**	expressed sequence AV026525	-3.05	-1.12	-4.73	-1.08	-1.10	-1.02	2.32E-08	1.78E-01	2.10E-10	3.05E-01	2.76E-01	7.91E-01
1422912_at	**Bmp4**	bone morphogenetic protein 4	-5.20	-1.37	-5.55	-1.01	-1.59	-1.83	5.98E-10	3.02E-03	1.19E-10	9.15E-01	1.70E-04	5.55E-06
1453651_a_at	**Brp44l**	brain protein 44-like	-1.34	-1.33	-4.84	-1.20	2.66	-1.53	1.15E-03	1.22E-03	2.71E-11	1.08E-02	1.46E-08	2.10E-05
1418507_s_at	**Cish2/Socs2**	cytokine inducible SH2-containing protein 2	-4.01	-1.17	-4.83	-1.05	-1.48	-1.16	4.11E-11	1.41E-02	3.10E-12	3.20E-01	1.77E-05	1.06E-02
1449109_at	**Cish2/Socs2**	cytokine inducible SH2-containing protein 2	-4.14	-1.25	-5.11	-1.14	-1.33	-1.26	9.53E-10	1.16E-02	6.43E-11	7.59E-02	2.84E-03	5.63E-03
1419430_at	**Cyp26a1**	cytochrome P450, 26, retinoic acid	-5.75	-1.27	-6.49	-1.16	-1.07	-1.84	2.38E-09	3.91E-02	3.52E-10	1.32E-01	4.98E-01	2.96E-05

1428834_at	**DUSP4**	dual specificity phosphatase 4	-3.02	-1.51	-4.03	-1.49	-1.41	-1.61	1.31E-07	1.03E-03	3.66E-09	5.37E-04	3.74E-03	1.34E-04
1415834_at	**Dusp6**	dual specificity phosphatase 6	-6.84	-1.07	-11.20	-1.46	-1.31	2.53	1.31E-08	6.31E-01	3.51E-10	7.96E-03	6.46E-02	6.58E-06
1426001_at	**eomes/Tbr2**	eomesodermin homolog	-1.67	-1.01	-3.99	-1.01	-1.16	-1.04	1.56E-06	8.64E-01	1.46E-11	8.26E-01	1.96E-02	4.72E-01
1435172_at	**Eomes/tbr2**	eomesodermin homolog	-1.58	-1.08	-3.62	-1.09	-1.30	-1.04	7.56E-08	5.20E-02	3.36E-13	1.88E-02	1.59E-05	2.77E-01
1436926_at	**Esrrb**	estrogen related receptor, beta	-3.03	-1.27	-6.31	-1.02	-1.02	-1.51	6.61E-09	5.94E-03	8.42E-12	7.48E-01	7.89E-01	4.13E-05
1428816_a_at	**Gata2**		-1.27	-1.57	4.04	-1.04	-1.17	-1.11	2.64E-02	4.64E-04	3.07E-09	6.35E-01	1.07E-01	2.14E-01
1450333_a_at	**Gata2**	GATA binding protein 2	-1.85	2.15	5.11	-1.13	-1.19	-1.50	2.02E-08	2.01E-09	1.72E-13	8.18E-03	2.39E-03	4.72E-07
1430357_at	**H3f3b**	H3 histone family 3B	-1.59	-1.33	4.46	-1.07	-2.63	-3.42	3.32E-05	1.55E-03	6.78E-11	2.92E-01	2.30E-08	5.60E-10
1454159_a_at	**Igfbp2**	insulin-like growth factor binding protein 2	3.74	-1.31	-1.39	-1.14	-1.28	-1.18	8.75E-11	5.64E-04	3.84E-05	2.33E-02	9.84E-04	5.99E-03
1418045_at	**Inpp1**	inositol polyphosphate-1-phosphatase	-3.52	-1.23	-7.12	-1.16	-1.06	-1.24	4.25E-09	2.02E-02	1.07E-11	5.76E-02	4.58E-01	8.45E-03

1423294_at	**Mest**	mesoderm specific transcript	-1.28	-1.28	-3.86	-1.09	-1.09	-1.43	1.35E-03	1.61E-03	3.52E-11	1.39E-01	1.80E-01	3.15E-05
1416298_at	**Mmp9**	matrix metallopeptidase 9/ gelatinase	-3.80	-1.62	-5.24	-1.14	-1.38	-1.11	3.12E-09	7.79E-05	9.30E-11	9.63E-02	1.81E-03	1.51E-01
1448291_at	**Mmp9**	matrix metalloproteinase 9	-2.78	-1.60	-6.73	-1.13	-1.28	-1.10	1.03E-06	9.40E-04	4.90E-10	2.27E-01	3.90E-02	3.56E-01
1422771_at	**mSmad6**	Smad6	-1.43	-1.19	-4.64	-1.19	-1.43	-1.14	4.61E-05	9.05E-03	5.03E-12	4.55E-03	5.27E-05	2.49E-02
1428942_at	**Mt2**	metallothionein 2	2.34	-1.01	4.60	-1.13	-1.17	-2.04	1.95E-09	8.13E-01	1.03E-12	1.38E-02	7.09E-03	3.87E-09
1436702_at	**NAT8L**	N-acteyltransferase 8-like	-3.25	-1.18	-3.53	-1.00	-1.13	-1.10	4.56E-09	4.11E-02	6.67E-10	9.72E-01	1.18E-01	1.68E-01
1450976_at	**Ndr1**	N-myc downstream regulated 1	-1.80	-1.33	4.32	-1.12	-1.41	-1.68	1.09E-04	1.61E-02	4.58E-09	2.28E-01	5.80E-03	1.22E-04
1456174_x_at	**Ndr1**	N-myc downstream regulated 1	2.18	-1.47	4.97	-1.14	-1.91	-2.03	1.62E-05	4.43E-03	3.66E-09	2.05E-01	8.32E-05	1.41E-05
1420760_s_at	**Ndrl**	N-myc downstream regulated-gene-1	2.15	-1.51	5.49	-1.15	-1.50	-2.00	6.78E-05	6.43E-03	8.36E-09	2.44E-01	7.53E-03	6.08E-05
1434270_at	**NPTXR**	pentraxin receptor	-2.43	-1.29	-4.67	-1.16	-1.42	-1.18	1.49E-06	2.31E-02	1.60E-09	1.06E-01	3.56E-03	7.61E-02

1417760_at	**Nr0b1**	nuclear receptor subfamily 0, group B, member 1	-8.49	-2.39	-8.46	-1.07	-1.90	-1.77	3.22E-12	4.90E-08	9.74E-13	2.53E-01	1.12E-06	1.19E-06
1428140_at	**Oxct**	3-oxoacid CoA transferase	-1.86	-1.48	-3.51	-1.07	-1.14	-1.14	8.26E-06	4.17E-04	1.95E-09	3.33E-01	1.29E-01	9.61E-02
1417837_at	**PHLDA2**	pleckstrin homology like domain, family A, member 2	-1.69	2.04	3.76	-1.12	-1.08	-1.39	1.01E-04	6.10E-06	3.68E-09	1.71E-01	4.10E-01	1.69E-03
1417553_at	**PLAC1**	placenta specific 1	-1.76	2.21	6.06	-1.11	-1.01	-1.62	1.09E-04	5.16E-06	3.48E-10	2.52E-01	9.29E-01	1.62E-04
1423933_a_at	**plet1**	placenta expressed transcript 1	-3.05	-1.04	-4.54	-1.07	-1.66	-1.60	1.04E-08	6.24E-01	1.22E-10	3.49E-01	2.72E-05	1.80E-05
1454254_s_at	**plet1**	placenta expressed transcript 1	-3.19	-1.14	-5.07	-1.05	-1.75	-1.62	1.22E-07	1.91E-01	1.10E-09	5.95E-01	1.26E-04	1.65E-04
1460038_at	**POU3F1**	POU domain class 3 transcription factor	-2.19	-1.02	-3.55	-1.21	-1.25	-1.27	5.27E-08	7.37E-01	9.82E-11	4.63E-03	3.56E-03	9.70E-04

1438671_at	**PPP2r2C**	protein phosphatase 2 regulatory subunit B, gamma isoform	-1.12	-1.33	-4.04	-1.03	-1.28	-1.10	1.23E-01	1.78E-03	1.69E-10	6.31E-01	4.77E-03	1.65E-01
1420664_s_at	**Procr**	protein C receptor, endothelial	-2.27	-1.20	-3.63	-1.12	-1.33	-1.42	1.04E-08	6.54E-03	2.41E-11	4.18E-02	2.67E-04	1.74E-05
1417928_at	**Ril-pending**	reversion induced LIM gene	-1.82	-1.08	3.76	-1.12	-1.15	-1.25	1.39E-05	3.46E-01	1.44E-09	1.56E-01	1.23E-01	1.05E-02
1423025_a_at	**Schip1**	schwannomin interacting protein 1	-1.72	-1.20	-4.01	-1.04	-1.20	-1.37	1.78E-06	1.02E-02	2.88E-11	4.77E-01	1.04E-02	9.30E-05
1436584_at	**Spry2**	sprouty homolog 2 (Drosophila)	-3.27	-1.14	-5.01	-1.30	-1.38	-1.33	7.60E-08	1.97E-01	9.21E-10	9.81E-03	5.58E-03	5.95E-03
1427072_at	**STARD8**	START domain containing 8	-1.27	-2.14	-4.23	-1.18	-1.37	-1.06	1.59E-02	2.03E-06	8.94E-10	5.11E-02	3.58E-03	4.42E-01
1451458_at	**TMEM2**	transmembrane protein 2	-2.25	-1.22	-3.68	-1.02	-1.16	-1.02	6.99E-07	3.37E-02	1.59E-09	8.29E-01	9.21E-02	7.47E-01
1417122_at	**VAV3**	vav 3 oncogene	-1.11	-1.07	3.56	-1.27	-1.37	-1.19	2.97E-01	4.98E-01	7.82E-09	1.43E-02	5.80E-03	5.53E-02
1456140_at	**Zic5**	zinc finger protein of the cerebellum 5	-3.41	-1.20	-5.00	-1.01	-1.10	-1.35	1.75E-09	2.16E-02	2.84E-11	9.01E-01	2.08E-01	5.20E-04
1456219_at	**Zic5**	Zic family member 5	-3.44	-1.19	-5.96	-1.02	-1.14	-1.47	1.16E-06	2.03E-01	8.36E-09	8.40E-01	3.22E-01	6.61E-03

1434705_at	**ZRANB1**	zinc finger RAN binding domain containing 1	-2.28	-1.00	-3.76	-1.17	-1.25	-1.25	1.41E-05	9.83E-01	4.23E-08	1.39E-01	7.25E-02	4.66E-02
1425887_at		RIKEN cDNA 4930511J11 gene	-2.72	-1.39	-3.78	-1.20	-1.02	-1.01	1.55E-08	5.21E-04	2.30E-10	1.37E-02	8.30E-01	8.26E-01
1427518_at		Mus musculus mRNA for zinc finger protein, partial cds	-2.11	-1.20	-3.57	-1.23	-1.06	-1.43	6.97E-07	3.46E-02	8.13E-10	1.02E-02	4.49E-01	2.05E-04
1439535_at		ESTs	-1.31	-1.19	3.77	-1.09	-2.04	-2.48	3.90E-03	4.05E-02	5.89E-10	2.44E-01	1.17E-06	3.28E-08
1448600_s_at		Unknown (protein for MGC:27838)	-1.10	-1.10	3.74	-1.25	-1.22	-1.20	2.31E-01	2.60E-01	8.98E-10	8.36E-03	2.58E-02	2.53E-02
1460121_at		ESTs, Highly similar to S12207 hypothetical protein [M.musculus]	-1.99	-1.80	4.00	-1.01	-1.05	-1.43	1.81E-07	8.64E-07	3.35E-11	8.40E-01	4.24E-01	3.77E-05

5.1.12.2 Real Time PCR validation of expression

Because microarray data is subject to many sources of variation (Affymetrix Inc., 2006b), and even with a low *p value,* over thousands of genes some of them would be expected to "change" simply due to chance. The gold standard has been verification of expression levels using quantitative PCR (Kuo *et al*., 2006). Using the annotated set of genes PCR primers were designed for some of the genes identified in each union that had been assigned a name and description. Primers were designed for all of the named genes identified as changed with 733 and 337 treatments, however not all primers designed were successful. The sequences were obtained from Entrez and Primers designed as described in 2.2.3.3.3. The contrast expression found via the microarray and the corresponding relative expression found using real time PCR for each gene investigated is shown below (tables 5.1-13-5.1-14).

In general the fold change found using real time PCR had a high correlation with the microarray results, and in fact many times a greater folds change was found. Only two genes measured had a fold change expression different from that measured using the microarray, and of these it was not for all of the different treatments. To test if genes which appeared to be down regulated with *Elf5* knockdown were up-regulated with *Elf5* over-expression, an *Elf5* expressing construct (pTracer-Elf5) was transiently transfected into mTS cells (section 2.2.1.7.4). This gave a 5 fold up-regulation in *Elf5*.expression twenty-four hours after transfection. The genes *Cyr61* and *Baz1a* which had shown a down regulation with *Elf5* knockdown, showed a significant up-regulation. The gene *Spi8* also showed a 1.5fold up regulation, however this was not significant.

Table 5.1-15: Validation of differentially expressed genes using real time PCR. The fold change difference measured using the microarray and by real-time PCR for selected in the union of the 733 siRNA treatment, 337 siRNA treatment and any of the growth factor experiments. Also included is relative expression of samples transiently transfected with an Elf5 construct compared to the blank control. Bold P<0.05, Bold and Italics P<0.01.

	Fold Change measured by the microarry					**Relative Expression measured using real time RT-PCR**						**Standard deviation of real time RT-PCR results (n=3)**						**Agreement**
Gene name	TSA vs N	TSF vs N	TS vs N	337 vs Si-	773 vs Si-	TSA/N	TSF/N	TS/N	337/Si-	733/Si-	elf5 over-expression/blank control	TSA/N	TSF/N	TS/N	337/Si-	733/Si-	elf5 over-expression/blank control	
CYR61	***-1.30***	***-1.56***	***-2.02***	***-1.60***	***-2.39***	-1.25	***-1.65***	***-3.43***	***-1.81***	***-3.52***	**1.40**	0.03	0.07	0.08		0.17	0.87	Y
SOX2	***-2.33***	-1.07	***-8.62***	***-1.44***	***-1.88***	***-2.53***	**-1.14**	***-11.69***	-1.30	***-1.62***	1.11	0.04	0.06	0.01	0.11	0.07	0.27	Y
ELF5	***-1.94***	***-1.17***	***-2.57***	***-2.07***	***-3.17***	**-2.01**	-1.18	***-3.12***	***-2.85***	***-4.15***	***5.53***	0.03	0.07	0.02	0.05	0.03	1.52	Y
HS3ST3B1	***-2.29***	-1.00	***-5.17***	***-1.76***	***-2.35***	-1.07	***2.07***	***-3.61***	***-2.67***	***-2.53***	1.01	0.14	0.35	0.08	0.06	0.09	0.46	Y for siRNA, N for GF
WDR40B	***-3.79***	***-1.30***	***-3.32***	***-1.43***	***-1.58***	***-4.02***	***-1.43***	***-4.66***	-1.26	***-1.61***	1.27	0.08	0.06	0.03	0.13	0.07	0.19	Y
SYNPO2L	***-1.34***	***-2.22***	***-4.17***	***-1.76***	***-2.01***	-1.25	***-1.69***	***-3.39***	**-1.68**	**-2.67**	1.29	0.13	0.07	0.04	0.14	0.06	0.23	Y

Table 5.1-16 Validation of differentially expressed genes using real time PCR. The fold change difference measured using the microarray and by real-time PCR for selected in the union of the 733 siRNA and 337 siRNA treatment however exclucding genes differentially expressed by growth factor removal. Also included is relative expression of samples transiently transfected with an Elf5 construct compared to the blank control. Bold P<0.05, Bold and Italics P<0.01.

	Fold Change measured by the microarray		**Relative Expression (real time PCR)**			**Standard deviation (n=3)**			
Gene Name	337 vs Si-	773 vs Si-	337/si	733/si	elf5 over-expression/blank control	337/si-	733/si-	Elf5 over-expression/blank control	Agreement
ALDH18A1	***-1.48***	***-1.61***	-1.72	-1.80	1.36	0.08	0.08	0.20	Y
BAZ1A	***-1.48***	***-1.74***	**-1.47**	**-1.54**	**1.64**	0.10	0.08	0.28	Y
CALCA	***-1.47***	***-1.63***	**-2.59**	-3.39	-1.14	0.18	0.13	0.21	Y
FBXO21	***-1.48***	***-1.70***	**-1.75**	**-1.77**	**1.23**	0.09	0.09	0.19	Y
FCHO2	-1.50	***-1.65***	-2.09	-1.51	1.29	0.09	0.28	0.34	Y
FSTL1	***-1.47***	***-1.74***	**-1.90**	***-2.00***	-1.02	0.21	0.09	0.27	Y
NARS	***-1.49***	***-1.74***	**-1.83**	**-2.05**	1.45	0.09	0.08	0.33	Y
PSAT1	***-1.61***	***-1.55***	**-1.83**	**-1.96**	-1.17	0.10	0.14	0.17	Y
PYCR1	***-1.47***	***-1.66***	-1.97	-2.53	1.01	0.08	0.06	0.25	Y
WNK3	***-1.48***	***-1.87***	-1.64	**-1.76**	-1.05	0.03	0.12	0.24	Y

Table 5.1-17: Validation of differentially expressed genes using real time PCR. The fold change difference measured using the microarray and by real-time PCR for selected genes in the union of the 733 siRNA and any of the growth factor experiments excluding genes differentially expressed by 337 siRNA treatment. . Also included is relative expression of samples transiently transfected with an Elf5 construct compared to the blank control. Bold P<0.05, Bold and Italics P<0.01.

	Fold Change measured by the microarray					**Relative Expression (real time PCR)**						**Standard deviation (n=3)**						Agreement
Gene Name	TSA vs N	TSF vs N	TS vs N	337 vs Si-	773 vs Si-	TSA /N	TSF /N	TS/ N	337 /Si	733 /Si	elf5 over-expression/ blank control	TSA /N	TSF /N	TS/ N	337/ Si-	733/ Si-	elf5 over-expression/ blank control	
ID3	***-1.18***	***2.20***	***2.01***	-1.06	***2.20***	-1.26	***4.18***	***3.67***	-1.04	***2.16***	-1.04	0.11	0.39	0.43	0.10	0.35	0.16	Y
DAB2	***2.55***	***-2.42***	-1.01	-1.07	***-1.88***	***2.97***	***-4.47***	1.11	1.01	***-2.43***	-1.06	0.29	0.02	0.12	0.08	0.10	0.23	Y
SERPINB8	**-1.22**	***-1.83***	***-2.06***	***-1.36***	***-1.88***	***1.51***	***-6.44***	***-8.46***	***-1.35***	***-4.63***	1.53	0.09	0.02	0.01	0.08	0.04	0.37	Y
HMGA2	***-2.15***	***-1.46***	***-1.62***	***-1.26***	***-2.18***	***-2.28***	-1.73	***-2.20***	**-1.34**	***-1.90***	1.23	0.03	0.28	0.04	0.05	0.10	0.28	Y
CSRP1	***2.00***	***-1.39***	***-1.96***	-1.00	***-1.78***	1.97	1.53	***1.75***	**1.01**	***1.50***	-1.01	0.22	0.13	0.13	0.11	0.35	0.18	N, only TSA agreed
WDR40B	***-3.13***	-1.20	***-3.65***	**-1.39**	***-1.73***	***-4.02***	***-1.43***	***-4.66***	-1.26	***-1.61***	1.27	0.08	0.06	0.03	0.13	0.07	0.19	Y

Table 5.1-18 Validation of differentially expressed genes using real time PCR. The fold change difference measured using the microarray and by real-time PCR for selected in genes differentially expressed by greater than 3.5 fold by removal of either Fgf4 or activin or both. Bold P<0.05, Bold and Italics P<0.01.

	Fold Change measured by the microarray			**Relative Expression (real time PCR)**			**Standard deviation (n=3)**			
Gene name	TSA vs N	TSF vs N	TS vs N	TSA/N	TSF/N	TS/N	TSA/ N	TSF/ N	TS/ N	Agreement
Ascl2	***-1.57***	-1.16	***4.17***	1.72	2.69	6.81	0.93	2.12	5.32	Y
Bmp4	***-5.20***	***-1.37***	***-5.55***	***-9.95***	**1.70**	***-10.86***	0.03	0.38	0.02	Y
Esrrb	***-3.03***	***-1.27***	***-6.31***	-2.42	-1.31	**-9.59**	0.07	0.05	0.03	Y
Gata2	**-1.27**	***-1.57***	***4.04***	1.41	**2.62**	4.28	0.71	0.84	2.34	Y
Mmp9	***-3.80***	***-1.62***	***-5.24***	***-2.78***	**-1.53**	***-8.64***	0.15	0.17	0.03	Y
Ndr1	***-1.80***	**-1.33**	***4.32***	2.11	**1.68**	**3.01**	0.49	0.27	0.52	Y, but an increase for Fgf4 removal,not a decrease

5.2 SUMMARY

In conclusion the microarray experiment has successfully identified genes which are differentially expressed upon Elf5 knockdown or removal of the growth factors essential for mTS cell maintenance. Although only small fold changes in differential gene expression were detected, especially in the samples treated with *Elf5* targeted siRNA, the genes differentially expressed appear valid as many are already known to be involved in placental development and real time PCR measurements show a strong correlation to the results obtained from the microarray.

CHAPTER 6 : DISCUSSION

6.1 TS CELL MODEL OF ELF5 TARGET GENES

Elf5 is essential for the development of the murine placenta; in particular, *Elf5* mutants did not develop an extra-embryonic ectoderm (ExE). The absence of the ExE precluded an in vivo subtractive approach for determining Elf5 target genes. We therefore used trophoblast stem (TS) cells as an in vitro model system. TS cells are derived from the polar trophectoderm of the blastocyst and appear to faithfully recapitulate the early in vivo development of the placenta requiring Fgf4 and Nodal signalling for their maintenance, thereby recapitulating the in vivo signalling from the epiblast to the adjacent polar trophectoderm(Erlebacher *et al.*, 2004; Tanaka *et al.*, 1998). Futhermore, TS cells express the same set of genetic markers as expressed by the polar trophectoderm and extra-embryonic ectoderm. TS cell differentiation can be induced by removing growth factor signalling (Fgf4 and/or Activin) whereupon they differentiate into the same downstream cell types seen after in vivo differentiation of the ExE (giant cells, syncytiotrophoblasts) (Hemberger *et al.*, 2004). TS cells have also been shown to be able to contribute to all cell types of the placenta in mouse chimeras (Tanaka et al., 1998). Therefore the in vitro study of Elf5 target genes in TS cells seemed warranted. The experimental approach consisted in modulating *Elf5* expression in TS cells and then measuring the resulting changes in gene expression to identify possible target genes. A similar approach has been used in identifying the target genes of other transcription factors such as the target genes of Oct4 in embryonic stem cells (Babaie et al., 2007; Matoba et al., 2006). Modulation of *Elf5* expression was attempted using two approaches: siRNA mediated silencing of *Elf5* and inducible over-expression of *Elf5*.

6.2 DEVELOPMENT OF AN *Elf5* INDUCIBLE TS CELL LINE

A TS cell line was unable to be developed which inducibly expressed the Elf5 gene using the TET On system (BD biosciences). This was because a TS cell line was unable to be established which expressed the rtTA protein in a position where it could inducibly express a luciferase reporter plasmid. The main reason for this is that a co-transfection was used in which the puromycin selection cassette was transfected at a

much higher molar ratio to the pTetOn vector carrying the rtTA gene than intended. Other effects such as cellular toxicity of the rtTA protein may have also played a role in the difficulty of establishing a cell line which expressed this protein in high amounts (Ackland-Berglund & Leib, 1995).

6.3 SIRNA MEDIATED KNOCK DOWN OF ELF5

Successful siRNA induced silencing of *Elf5* mRNA was achieved using two *Elf5* siRNA oligonucleotides of the four designed. The efficiency of *Elf5* knockdown was influenced by two main factors; the transfection efficiency using Lipofectamine 2000 (Invitrogen Life Technologies) and the efficacy of a particular siRNA sequence to direct knockdown of the *Elf5* mRNA target.

After the identification of the two siRNA oligonucleotides which gave knockdown the transfection efficiency was optimised. The main factor influencing transfection efficiency is the ability to transfect cells at a stage when they are healthy and proliferating at a maximum rate (Ausubel *et al.*, 1999). A standard protocol was established which allowed cells to be seeded the day before transfection and to be at the recommended confluency at transfection to minimise toxicity effects of the Lipofectamine. Experiments were harvested twenty four hours following siRNA transfection to give a greater chance of identifying direct target genes of the Elf5 transcription factor. Longer experimental timeframes would have allowed more secondary interactions to occur and influence the gene expression profile. TS cells are quite sensitive to perturbations in culturing conditions, leading to their differentiation. In fact a low level of spontaneous differentiation in the TS cell populations was always seen by the observance of giant cells. Precocious giant cell differentiation could be induced with over-pipetting the cell suspensions. A twenty four hour time period for the experiment was advantageous because it allowed seeding of the wells with TS cells prior to transfection at a confluency that was not too low, so they did not differentiate. It also meant that by the time the cells were harvested the day after transfection, they were at 80-90% confluency. A longer experimental time period would have resulted in over growth of the colonies and differentiation, so that the gene expression changes could be due to culture conditions and not siRNA treatment.

The best concentration of siRNA to use in a transfection was also optimised and consistently the knockdown rate of *Elf5* with the best siRNA (called 337) was greater

than 80%, although in one experiment a 95% reduction in *Elf5* expression was measured. Transfection with a fluorescently labelled siRNA oligonucleotide also showed a high level of transfection efficiency; green fluorescence could be seen in more than 80% of cells twenty four hours following transfection. The establishment of a high knockdown of *Elf5* was important in order to detect downstream targets. Mice heterozygouse for the *Elf5* mutant allele are normal and viable (Donnison et al., 2005; Zhou et al., 2005) indicating a high level of *Elf5* reduction is required to see a phenotypic effect. Additionally, because the entire population of TS cells was analysed for changes in gene expression, changes in individual cells as a result of successful transfection would be averaged across the gene expression profile of all the cells in the experimental well. Despite the standard protocol established, siRNA knockdown was still variable. This is due to the variable transfection efficiency. Errors in cell counting and seeding of the wells, passage number at transfection, and quality of the conditioned medium, all influence cell health and proliferative ability. Therefore following each transfection the exact knockdown of *Elf5* had to ascertained compared to a siRNA negative control treated well. This was particularly important in generation of samples for measurement of candidate gene and microarray analysis. Expression of *Elf5* in the siRNA treated samples was measured using reverse-transcription real time PCR (RT-PCR).

6.3.1 Real time PCR Measurement of Gene Expression

The extraction of RNA, in order to measure the gene expression of *Elf5* targeted siRNA treated cells relative to controls, involved many steps which could all be sources of error and therefore influence the absolute expression of a gene measured. An in house protocol was used which was based on the TRIZOL (Invitrogen Life Technologies) extraction method. However during the course of this work two problems were found with using this protocol.

Firstly there was a large amount of variation in the Ct measured of the housekeeping gene, indicative of RNA being lost or degraded during the isolation process. RNase contamination is very easy to get as RNAse enzymes are able to spontaneously reform their active state even following processes such as autoclaving. They are also very abundant, as they are found in all organisms including bacteria. The best defense against RNases is to remove all sources of contamination (Sambrook & Russel, 2001b). Using RNAse free technique the primary source of contamination is the RNA source

itself. The TRIZOL product insert advised applying 1ml of TRIZOL directly to cells while still in their dishes. This is because Trypsin enzyme can be a source of RNase contamination and adding TRIZOL immediately to the samples prevents the action of endogenous RNAses (Invitrogen life technologies, 2003). Previously 400µl of TRIZOL had been added to the cell pellet in a minicentrifuge tube following harvesting with the recombinant Trypsin enzyme. Following these changes a consistent level of total RNA was isolated from all samples in an experiment. These results indicate the Trypsin enzyme had been a possible source of contamination and the amount of RNAse inhibitory guanidinium salts (in the TRIZOL) was unable to overcome this.

Secondly, the reverse transcription negative control samples for some RNA extractions had a Ct value very close to that of the corresponding cDNA sample. This indicated DNA contamination, and an inefficient DNAse step. Following concentration analysis of total RNA it was obvious the amounts of total RNA and correspondingly DNA were too high for the amount of DNAse enzyme used. The DNAse step was consequentially altered to use a four fold greater amount of enzyme and the incubation time doubled. The laboratory protocol previously used was a generic one that was also used for isolation of total RNA from embryos; it was obviously not adapted to procedures isolating large amounts of RNA such as the experiments performed here.

During the course of this work a number of changes were also made to the methods in calculating the relative expression of a gene of interest in an *Elf5* targeted siRNA treated sample compared to a control treated sample (section 2.2.3.3). Real time PCR analysis of gene expression was particularly suitable in this case because it allowed the sensitive detection of changes in gene expression of as little as 25% (Gentle et al., 2001). Relative expression calculations were based on the $2^{-\Delta\Delta Ct}$ method which uses a housekeeping gene to firstly normalise a sample before expressing it relative to the control (Livak & Schmittgen, 2001). The first changes were in how the amplification efficiency was calculated. Using the $2^{-\Delta\Delta Ct}$ an amplification efficiency of 2 is assumed for both the gene of interest and the housekeeping gene. The amplification efficiency has a large impact on the normalised copy number calculated. For example, a 5% difference can make one gene appear twice as abundant after 26 cycles (Freeman et al., 1999). To overcome this, four serial dilutions of one of the samples being analysed were also measured for each primer set used. Efficiency was then calculated for a

particular primer set for each run by graphing the Cts of the dilution series. The calculated amplifications were incorporated into the relative expression calculations in place of '2'. Although this was more accurate it still had problems; the amplification efficiency of one sample may not represent the amplification of all samples due to the presence of different levels of inhibitory compounds in different samples (such as DTT used during reverse transcription reactions). A line of best fit was used to calculate the efficiency. However, the points were often not exactly linear, especially at higher dilutions. For real time PCR validation of the microarray results a third method of calculation was used. Expression levels were quantitated on the Rotor gene 6000 which allows an individual measurement of amplification efficiency per sample. This is a more accurate way of obtaining the amplification efficiency (Tichopad *et al.*, 2003; Wong & Medrano, 2005).

6.3.2 Effects on TS Cells of siRNA Mediated Elf5 Knockdown

6.3.2.1 No Interferon Response was Induced

One of the reported effects of using RNAi to reduce gene expression is the induction of an interferon immune response (Bridge et al., 2003). This is a natural reaction of a cell to dsRNA introduced in a viral infection and can cause changes in gene expression to be measured which are not as a result of changes in the target gene expression. To check this was not the case, two genes from the viral responsive group of Interferons were measured, *Interferon-a4* and *Interferon-β*, (Samuel, 2001) using real-time PCR for induction. Interferon expression of 337 siRNA treated cells compared to non-treated cells showed no difference in interferon expression levels. Although an interferon response due to the 733 siRNA was not measured, later analysis in the microarray experiment confirmed a lack of interferon response as in the set of top twenty functional groups affected by both *Elf5* siRNAs the immune response functional group was not present.

6.3.2.2 No Changes in Cell Morphology/DNA Content as an Indication of TS Cell Differentiation

Upon differentiation of TS cells giant cells are commonly formed (Tanaka et al., 1998). These cells are characterised by their large size and by the fact they replicate their DNA without undergoing mitosis to become polyploid (Simmons et al., 2007). Because Elf5

is essential for maintaining the proliferative ability of extra-embryonic cell precursors, it was hypothesised that a reduction of Elf5 could lead to increased differentiation of the TS cells. Casual observation the day after a transfection indicated this was not the case. This was later confirmed by flow cytometry which showed no overall increase in the DNA content per cell in a population of *Elf5* siRNA treated TS cells. The reason for this is likely to be that the timeframe for morphological changes to occur was too short. Even upon complete growth factor withdrawal TS cells do not morphologically differentiate into giant cells until about four days later (Erlebacher et al., 2004).

6.3.3 Candidate Gene Expression Changes as a Result of Elf5 Silencing

Following successful knockdown of Elf5, some of these samples were analysed for changes in the expression of genes known to have important roles in trophoblast differentiation *in vivo*. Apart from possible changes in expression of the pro-protein protease Furin, a regulator of Nodal signalling (Beck et al., 2002), and *Eomes* no significant changes in gene expression were observed as a result of changes in *Elf5* expression (Figure 3.3-2). Changes were however observed in the expression of some of the candidate genes (*Bmp4*, *Errβ, Eomes, Fgfr2* and *Tcfap2c*) when the growth factors were removed. Some of the genes were not changed even with growth factor removal in the twenty four hour time period (*Hand1, Cdx2, Stra13* and *Spc4*). The lack of genes which were significantly changed when analysed by candidate gene analysis was later confirmed with the microarray. All of the genes tested previously in the candidate gene analysis also showed no change in expression due to *Elf5* silencing, including *Furin* and *Eomes*. There was a perfect correlation between those genes seen to go up or down in expression with growth factor removal between the microarray results and the real time PCR candidate gene analysis results.

6.4 MICROARRAY ANALYSIS

6.4.1 Microarray Quality Analysis

6.4.1.1 Technical Quality of RNA Samples and Arrays

Before microarray analysis was carried out, RNA samples to measure changes in gene expression as a result of Elf5 silencing or growth factor removal were rigorously tested

for quality as well as for Elf5 knockdown. This is because the greatest source of controllable variability is in the quality of the RNA labelled (Affymetrix Inc., 2006b) Following labelling and hybridisation of the RNA samples, several control parameters of the microarrays were analysed to ensure the experiment had been technically successful. All control parameters were found to be within the limits advised by the manufacturers (Affymetrix).

6.4.1.2 Biological Validation

To ensure the results of the array were biologically relevant some genes observed as significantly changed via the microarray were also analysed using real time PCR. Biological validation of the array was done by analysing a selection of the genes that had been identified as changed on the microarray by real-time PCR. There was a close correlation between the fold change (up or down) measured using the microarray and using real-time PCR. An important observation was that the top two probe sets changed in arrays treated with either *Elf5* targeted siRNA (733 or 337) mapped to *Elf5*. Annotation of probe sets significantly changed for gene name revealed a number of them were known to be involved in TS cell differentiation. This was especially the case with the set of 57 genes changed by greater than 3-fold upon growth factor withdrawal. This set included genes such as matrix metalloproteinase 9 (*Mmp9*), *Ascl2, Errβ, Tcfap2c* and *Eomes* and all of which are known to be expressed in derivatives of the polar trophectoderm (Auman *et al.*, 2002; Luo et al., 1997; Russ et al., 2000; Scott et al., 2000; Sharma, 1998).

6.4.2 Global Analysis Results

The Log_2 expression per probe set between samples treated with 337 or 733 *Elf5* targeted siRNA and the siRNA control treated samples showed a high correlation (0.998 and 0.994 respectively). This indicates that overall the majority of genes were unchanged by *Elf5* knockdown over the time period. The 733 siRNA treatment had a greater effect on global gene expression than 337 siRNA treatment, as shown by the slightly lower correlation between 733 siRNA treated samples and siRNA control treated samples. This result was confirmed by the fact that the Elf5 microarray probe sets showed a 3 fold change in 733 siRNA treated samples versus a 2 fold change in 337 siRNA treated samples. The greater knockdown of Elf5 by the 733 siRNA oligo came as a surprise and was contrary to previous results whereby 337 siRNA induced

knockdown had always been more successful (section 3.2.2.2). *Elf5* real time PCR analysis using the Rotor gene 6000 (Corbett) on three of the same samples used in the microarray analysis also showed the same result; Elf5 was down regulated over 4 fold in 733 siRNA treated samples compared to 2.9 fold in 337 treated samples. The 337 and 733 transfection experiments were carried out at separate times. The health and proliferation ability of cells is the biggest influence on transfection efficiency (Ausubel *et al.*, 1999). Although cells at the same passage number were used for the different experiments and a standard seeding protocol was used. It is quite likely that factors such as quality of the conditioned medium used and previous handling of the cells (such as pipetting too vigorously) could have affected cell health and therefore growth and transfection efficiency. This could have contributed to the poorer than usual knockdown seen in the 337 siRNA treated samples.

Log_2 expression correlation between each of the growth factor treatments versus the non-treated normal cells also showed high correlation. As expected TS cells grown without both Fgf4 and Activin had the lowest correlation with cells grown in normal medium (0.983). Removal of Fgf4 had a much greater overall effect on gene expression than Activin removal; the correlations were 0.991 and 0.996 respectively. This suggests Fgf4 signalling has a greater number of target genes than Activin signalling. Erlebacher *et al* (2004) observed a greater rate of differentiation when the Activin/Tgfβ source (conditioned medium) was removed along with the addition of anti-Tgfβ neutralising antibodies than just removing the conditioned medium alone. This implies another source of Tgfβ was present in the culture conditions apart from that supplied by the conditioned medium (Erlebacher *et al.*, 2004). The authors suggest the source is a combination between that produced by the trophoblast cells themselves and from the (20%) fetal calf serum used in the medium. The culture system used in this experiment was very similar to that used by Erlebacher *et al* (2004); a concentration of 20% fetal calf serum was also used in the medium. Therefore other sources of Tgfβ could be compensating for the activin removal, giving only small changes in gene expression.

A plot of Log_2 expression of the normal treated sample versus the siRNA negative control treated sample showed a high correlation of 0.995. This is important because it indicates that treatment simply due to siRNA treatment/transfection with Lipofectamine 2000 had little effect on the gene expression of the cells. The high correlation was also significant because as mentioned above experiments were carried

out at different time points for practical reasons. Also in order to get high quality RNA some transfection experiments had to be repeated a number of times. This was particularly the case with the 733 siRNA transfected samples which were collected months after the first samples. Changes in the conditioned medium quality and cell handling could cause subtle changes in gene expression over time, so some genes could appear differentially expressed as an artefact. The siRNA negative control treated sample was harvested at the same time as the collected 337 siRNA treated samples. To counteract this and the effects of the transfection procedure on the TS cells, genes which were differentially expressed between siRNA negative control treated samples and the normal treated samples were discarded from the set of genes identified as being changed using the Elf5 directed siRNA oligonucleotides.

6.4.3 Identification of Genes of Interest

Arbitrary fold change cutoffs were applied to the Log_2 contrast value (section 2.2.5.7). for each probe set between a treatment and its relative control (siRNA control or normal growth medium control) to obtain a set of probes of interest. The *p* values for the probes identified as significantly changed were inspected to ensure they were significant (<0.05). Using a *p* value alone as a measurement of significance would have increased the rate of false positives (genes identified as having changed by chance alone). This is because the standard *p* value for biological significance is 0.05, meaning 1 out of 20 probe-sets would be incorrectly identified as 'significantly' changed. Over the 45,101 probe sets in the Affymetrix mouse genome expression chip, many genes would be wrongly selected. The fold change cut offs selected were chosen purely in order to obtain a manageable set of genes changed for each treatment. The fold change cut offs were 1.4 fold for 337 siRNA treated samples, 1.5 fold for 733 siRNA treated samples and 2 fold for any of the growth factor treated samples.

6.5 BIOLOGICAL MEANING

Intersections of treatments were carried out, to identify the set of genes affected by siRNA treatment using either 733 or 337 siRNA. Because the genes in this set are changed by both siRNAs, the chance of them being differentially expressed due to the off target effects of one siRNA is small. A total of 22 probes were identified in this set. The set could be further divided into those probes (9) which were also differentially expressed by greater than 2 fold with growth factor removal. A pathway analysis of the

probes identified as significantly changed in either 337 or 733 siRNA treatments showed TgFβ signalling is an important part of the network. This is as would be expected because Tgfβ signalling is required for TS cell maintenance (Erlebacher et al., 2004). This indicates overlapping networks between genes regulated by *Elf5* and genes involved in TGFβ mediated stem cell maintenance.

Consistent with previous results showing Elf5 is regulated by Fgf4 signalling (Donnison *et al.*, 2005; Metzger *et al.*, 2007), Elf5 was found to be significantly down regulated in samples grown in medium without Fgf4 (2 fold down regulation). However, it was less affected by Activin removal, showing only a slight down regulation of 1.25 fold.

6.5.1.1 Possible Elf5 and Growth Factor regulated genes

The set of probes identified as the most interesting were those changed by *Elf5* siRNA treatment with either 337 or 733 siRNAs and growth factor removal. Nine probe sets fell within this set and they corresponded to the genes *Elf5*, *Cyr61*, *Sox2*, *Hst3st3b1*, *Wdr40b*, *Synop2l* and a probe set unable to be matched (using Ingenuity Pathway Analysis Software). The biological soundness of these genes is highest because they are changed by both siRNAs (thereby removing the chance they are changed due to non-specific RNAi effects (Echeverri *et al.*, 2006). It is envisioned this set of genes are common members in both Elf5 mediated TS cell renewal and growth factor induced TS cell renewal. The expression of all genes identified in this set was validated by real time PCR. The relative fold change up or down regulation measured by real-time PCR quantification agreed with the microarray results for all of these genes except *Wdr40b*, which was found not to change significantly in the 337 siRNA treated samples.

Two independent probe sets for cysteine rich angiogenic inducer 61 (*Cyr61*) were identified as significantly changed in the intersection between 337/733 siRNA treatment and all growth factors. This gene is associated with Insulin growth factor 1 (IGF-1) signalling and has been shown to augment growth factor induced DNA synthesis in endothelial cells (Ingenuity Pathway Analysis annotation). Aberrant expression of *Cyr61* is associated with breast cancer, wound healing, cell adhesion and migration (Mo *et al.*, 2002). Of most interest is the fact *Cyr61* homozygous knockout mice die as a result of failure in placental development, in particular chorioallantioic fusion and vascularisation of the placenta (Mo *et al.*, 2002). This information gives

soundness to the result from the microarray that Cyr61 is expressed in TS cells and down-regulated with differentiation or *Elf5* knockdown. Perhaps, like endothelial cells, *Cyr61* plays a role in the proliferation of the TS cells as a result of Elf5 transcription factor activity and Fgf4/Activin growth factor signalling.

The finding that *Sox2* was down regulated in this intersection was interesting as *Sox2* is a transcription factor associated with pluripotency cell lineages and is essential for the development of the placenta (Avilion *et al.*, 2003). *Sox2* is expressed in the same pattern as the well known pluripotency transcription factor, *Oct4*. However, it is also expressed in the multipotent cell lines of the extra-embryonic ectoderm. *Sox2* homozygous mutant embryos fail just after implantation, with a failure to maintain the ICM/epiblast. Wild type ES cells (which contribute only to the ICM) used to make a chimera could only rescue embryos until day 7.5, when they died due a failure to develop the chorion. Hence, TS cells were unable to be derived from *Sox2* homozygote knockouts, indicating *Sox2* plays a role in the proliferation and development of the extra-embryonic ectoderm (Avilion *et al.*, 2003).

Hst3st3b1 is one of the variants of heparin sulphate 3-O-sulfotransferase enzymes; these enzymes produce anticoagulant heparin sulphate and the particular form here (3b) is highly expressed in liver and placental tissue (Shworak *et al.*, 1999). A mouse knockout of Hst3st caused intrauterine growth retardation; pups born were smaller and survived only a few days after birth. Contrary to expectation blood clotting was not the reason for death and placental tissue was found to be histologically normal (Shworak *et al.*, 2002). More recently Hst3b1 has been shown to be involved in Notch signalling, which is a key gene in many developmental pathways (Kamimura *et al.*, 2004). From the microarray data and real-time PCR data, Hst3st3b1 was significantly down regulated by Elf5 knockdown. It is also down regulated by Fgf4 removal; this is an interesting result and may indicate Hst3st3b1 also plays a role in placental development.

Very little information is known about Synaptopodin 2-like (*Synpo2l*), so it is difficult to speculate its role in TS cells. It is only known that it is part of the cytoskeleton and is upregulated in human colorectal cancer (Nakamura *et al.*, 2004). Unlike the other genes in the 733/337 and growth factor intersection, *Synpo2l* was the only gene

significantly down regulated by Activin removal alone; this result was confirmed by real-time PCR to be significant. Wdr40b is a member of the large WD-repeat protein family (so far it has 126 members in humans). These proteins all share four or more repeating units of a 40 amino acid repeat (Li & Roberts, 2001). The functions identified for this family vary widely and include signal transduction, transcription regulation, cell cycle control and apoptosis. However, they are all mediated through protein-protein interactions (Li & Roberts). There is very little known information about *Wdr40b*, and this gene could be playing any of one of the above roles in TS cells.

The second intersection of greatest interest was the set 13 probes differentially expressed upon treatment with either Elf5 targeted siRNA but not significantly affected by growth factor removal. Among this set was Follistatin (*Fstl*). A negative regulator of Activin signalling, it binds tightly to Activin preventing it from interacting with Activin type II receptors (Shimonaka *et al.*, 1991). *Elf5* reduction also leads to a reduction in *Fstl* transcription, perhaps increasing the cells' susceptibility to Activin signalling. Vasorin binds to Tgfβ and is expressed in human placenta (Ikeda et al., 2004). However, in this experiment Vasorin was not significantly changed by Activin signalling and instead was identified as changed through siRNA mediated Elf5 knockdown. Baz1a is expressed in embryonic stem cells and is involved in ATP dependent chromatin remodelling (Bozhenok *et al.*, 2002). It is perhaps a common stem cell gene required for maintenance and proliferation of TS and ES stem cells.

Expressed sequence AU015836 is a cDNA sequence which from homology to a C. elegans protein (28%) and a hypotheticial *S. cerevisae* protein is predicted to also code for a protein. Interestingly cDNA fragments of this sequence have been identified in several cDNA libraries, including libraries created from whole mouse embryos E6.5-11.5, mouse embryonic germ cells, enriched cDNA from a 1 cell embryo, mouse embryonic stem cells and extraembryonic trophoblast tissue (source: Unigene; http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Mm&CID=328014 accessed 5/9/07). These observations indicate it could play a role in embryonic development.

6.5.1.2 Genes Identified as Being Changed upon Growth Factor Withdrawal

Annotation of the set of probes changed by greater than 3.5 fold upon growth factor removal (and therefore induction of TS cell differentiation) revealed several genes

which have previously been reported to be expressed in early placental tissue. In particular placenta expressed1 (*Plac-1*) was seen to increase 6 fold when mTS cells where induced to differentiate by removal of both Fgf4 and Activin. *Plac-1* is specifically detected in the trophoblast cell types of the EPC, giant cells and labyrinth cells from embryonic day 7.5 to 14.5 (Cocchia *et al.*, 2000). It is upregulated upon differentiation to the syncytiotrophoblast fate in human placenta (Massabbal *et al.*, 2005).

It was interesting to note that *Socs2* was one of the genes identified as down regulated in mTS cells when Fgf4 growth factor was removed. Both *Socs2* and *Elf5* are known to be essential for mammary gland development by playing key roles in prolactin signalling (Harris *et al.*, 2006). However, the *Elf5* siRNA treated samples did not show a significant down regulation in *Socs2* expression, so it seems *Elf5* does not directly regulate *Socs2*.

Consistent with the process of TS cell differentiation to giant cells, *Dusp4* and *Dusp6* were significantly down regulated on Fgf4 removal; these genes are phosphatases and negatively regulate MAPK/ERK family which are associated with cellular proliferation.

Gata2 was an interesting gene; unlike most genes it showed a significant up-regulation upon growth factor removal, and it appeared more affected by Activin removal (+1.8 fold) than Fgf4 removal (1.5 fold). It was up regulated 4.5 fold on removal of both Fgf4 and Activin showing a synergistic effect. *Gata2* expression is essential for haematopoietic stem cells and has been described as a "gatekeeper" to maintain their immaturity, with down regulation leading to differentiation (Minegishi *et al.*, 2003). *Gata2* is also known to bind alongside the Ets transcription factor *Elf1* to the enhancer of the *SCL* gene, whose expression is essential for the initiation of haematopoiesis and the formation of Haematopoietic stem cells (Gottgens *et al.*, 2002).

Mmp9 is a peptidase enzyme which degrades collagen and is involved in migration, invasion and proliferation. Its expression and involvement in mediating placental invasion has been extensively studied in both human and mouse trophoblast tissue (Sharma, 1998; Staun-Ram *et al.*, 2004).

Ndrg1 was down-regulated 8 fold upon loss of Fgf4, indicating it is very sensitive to changes in this signalling pathway. *Ndrg1* has been shown to be expressed in the early embryo in both the trophectoderm and in the derived trophoblast cell types. Under hypoxia expression of this gene is up-regulated (Clipsham *et al.*, 2004). In ES cells knockdown of *Ndrg1* leads to differentiation. In combination with the results from this microarray experiment which show Ndrg1 down regulation upon Fgf4 removal induced differentiation this indicates this gene is a common element to maintaining stem cell pluripotentcy.

In a similar experiment the effects of withdrawal of either Fgf4 or TGF-β/activin was investigated in mTS cells by monitoring the expression of the genes *Eomes*, *Errβ*, *Cdx2*, *Mmp9*, *Id2* and *Fgfr2* (Erlebacher et al., 2004). In agreement with the results found here, over the three day period following growth factor withdrawal there was a rapid down-regulation of *Eomes* and *Errβ* due to Fgf4 withdrawal, and less so to Activin withdrawal. Erlebacher *et al* (2004) observed a similar decrease in *Mmp9* and *Cdx2* expression with either Fgf4 or Activin withdrawal. In this experiment *Mmp9* was also found to be significantly down-regulated, however, to a greater extent with Fgf4 withdrawal. In contrast to Erlebacher *et al*'s (2004) findings, Cdx2 was not significantly down-regulated.

Retinoblastoma tumour suppressor gene (*Rb*) has recently been shown to play a critical role in TS cells (Wenzel *et al.*, 2007). In contrast to this study *Rb* was not found to be significantly changed (using microarray analysis). The greatest change seen was a 1.3 fold reduction in 733 treated cells which was smaller than the 1.5 fold cut off. This may be due to the short time period after measurement. However, *Rb* does not appear to be a direct target of Elf5.

6.5.1.3 Improvements/problems:

A major disadvantage of the experimental system used when *Elf5* knockdown was being studied was that the cells used for transcriptional analysis came from a population of cells which would all have been transfected to different degrees and therefore all have different levels of knock-down. This meant the overall knockdown measured was reduced, compared to a population of cells which had all received the

knock down treatment. Consequently, the downstream effects of the knockdown were also diluted out by the overall population, and it was this effect which meant overall fold changes of *Elf5* as measured by the microarray were a maximum of 2.3 fold for the 337 treated samples and 3.2 fold for the 733 treated samples. This resulted in the next biggest change in a genes expression of 1.8 fold for 337 and 2.6 fold for 733. Another factor in the low level of fold change was that the analysis was carried out after only twenty four hours; a longer period could have given greater fold changes. However there would have been the confounding factor that the siRNA transfection was only transient and so therefore the knockdown could not be maintained over longer periods of time.

In a similar paper to measure downstream targets of the Oct4 transcription factor in ES cells, a $Oct4^{+/-}$ cell line was used which could inducible express Oct4 when tetracycline was removed from the medium. In this experiment the fold change in *Oct4* after one day was just 1.2-fold at day two and 2-fold at day 3. Using the siRNA approach gave a similar fold change as these results. They also had log ratio changes which varied between -2 and +2. Therefore, although the changes appeared small they are similar to other experiments altering transcription factor expression.

In studies with a similar approach, using siRNA to reduce *Oct4* expression was followed by global microarray analysis; 36% of probes were down regulated. This is contrary to the results from this experiment, where the majority of probe sets found to be differentially expressed were down regulated (68%) as cells lost their stemness. *Oct4* is a transcription factor required for ES pluripotency, and a change of 50% of Oct4 in embryonic stem cells induces differentiation.

Validation of the microarray results was carried out on cDNA generated from the same samples used in the microarray experiment. This could increase the false discovery rate because identified changes in gene expression could be due to anomalies from a particular experiment, and not indicative of the result when carried out in a different experiment (Allison et al., 2006). It is therefore recommended that validation of microarray results should be carried out using new biological cases. This effect is mitigated to some degree, however, by the fact three samples of each treatment were used for validation, and each can be considered a separate biological case (they are each transfected and treated separately), so the chance all three are anomalies is low.

Concordance of the microarray results with the results from the candidate gene expression analysis, which was carried out on samples generated in a separate experiment, support the soundness of the findings. In future it would be better to generate a new set of samples for validation.

6.6 FURTHER RESEARCH

The targets identified as possible Elf5 targets need to be further investigated to show they are expressed at the correct time and place for Elf5 regulation. This could be achieved using *in situ* hybridisation on mouse embryos which identifies the location and time a transcript is expressed. Another confirmation of target genes is to use the recently published Elf5 DNA binding sequence: (T/a)A(T/a)AAGGAAGT(A/t)(A/t) (Choi and Sinha, 2006) for *in silico* analysis. The sequences within or near the possible target genes can be searched using bioinformatics algorithms to see if they contain conserved enhancer binding regions of DNA coding for the consensus *Elf5* DNA binding site.

Once the spatio-temporal expression of the potential target gene has been confirmed to coincide with *Elf5* expression, the role of that gene could also be investigated (for example, by generating a knockout mouse) and therefore by using Elf5 as a starting point the molecular networks underlying early placental development may be elucidated. This is of particular importance given the placenta's role in a variety of functions all essential to an embryo's survival.

Studies have shown that the over-expression of certain transcription factors such as Cdx2 is enough to drive ES cells into the trophectoderm lineage (Niwa *et al.*, 2005; Tolkunova *et al.*, 2006). It would be interesting to see if over-expression of Elf5 in ES cells has the same effect.

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APPENDICES

SEQUENCING OF PTRE-ELF5 DATA

A-1: With the poly-a globin primer

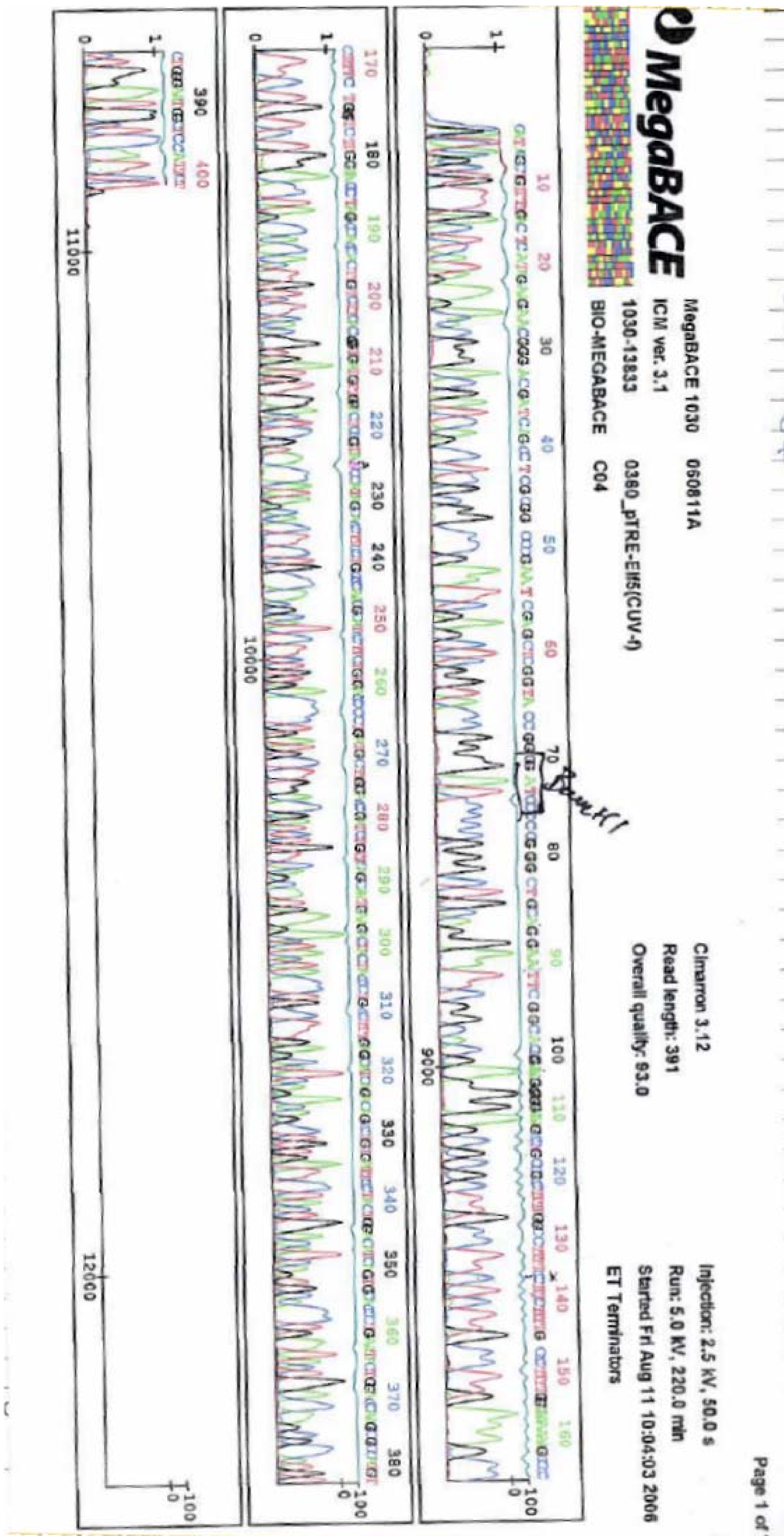
MegaBACE 1030 060811A
ICM ver. 3.1
1030-13833 0360_pTRE-Elf5(pA-glob-r)
BIO-MEGABACE D04

Cimarron 3.12
Read length: 412
Overall quality: 87.0

Injection: 2.5 kV, 50.0 s
Run: 5.0 kV, 220.0 min
Started Fri Aug 11 10:04:03 2006
ET Terminators

Page 1 of

A-2: Sequencing of the ptre-elf5 constructWith the CMV promoter Primer



A-3: Summary of the quality analysis performed on RNA samples prior to microarray analyses.

Samples were analysed for concentration and purity absorbance ratios on the nanodrop (Nanodrop Technologies). The absolute *Gapdh* Ct was measured on the ABI real-time PCR machine. *Elf5* and *Eomes* expression levels were normalised to Gapdh before being expressed relative to the siRNA negative or normal medium control. TS = Trophoblast stem cell medium, Fgf4 = fibroblast growth factor 4, Normal= TS cells grown in medium containing both Fgf4 and Activin.

Sample Name	Harvesting method	Concentration of total RNA ng/µl	Absorbance Ratio 260/280	RT-Gapdh Ct	Absolute Gapdh Ct (1/10 dilution of cDNA)	Relative Elf5 expression (to avg siRNA negative)	SEM	Relative Eomes expression (to avg normal)	SEM
TS + Fgf4 1	TRIZOL	1734	2.04	25.81	17.71			0.766	0.04
TS + Fgf4 2	TRIZOL	2797	1.97	27.01	17.17			0.774	0.04
TS + Fgf4 3	TRIZOL	2881	1.97	25.98	17.13			0.865	0.04
TS + Fgf4 4	TRIZOL	2798	1.99	26.96	16.94			0.796	0.04
TS + Fgf4 5	TRIZOL	2516	2.01	26.18	17.24			1.010	0.04
TS + Fgf4 6	TRIZOL	3271	1.90	26.48				0.000	0.04
TS + Fgf4 7	TRIZOL	2011	2.04	33.40	16.32			1.006	0.04
TS + Fgf4 8	TRIZOL	2079	1.98	28.23	18.03			1.050	0.04
TS + Fgf4 9	TRIZOL	1676	2.04	25.97	18.01			0.960	0.04
TS + Fgf4 10	TRIZOL	1663	2.04	26.00	18.21			1.121	0.04
TS + Fgf4 11	TRIZOL	1883	2.01	>35	18.25			1.077	0.04
TS + Fgf4 12	TRIZOL	1892	2.04	>35	17.92			0.916	0.04
TS + activin 1	TRIZOL	2018	2.04	32.37	17.27			0.515	0.01
TS + activin 2	TRIZOL	2070	2.04	24.34	17.20			0.537	0.01
TS + activin 3	TRIZOL	2069	2.06	31.92	17.53			0.538	0.01
TS + activin 4	TRIZOL	2246	2.03	25.63	17.29			0.533	0.01
TS + activin 5	TRIZOL	2653	2.00	30.25	17.03			0.571	0.01
TS + activin 6	TRIZOL	1215	2.04	27.26	18.32			0.580	0.01
TS + activin 7	TRIZOL	1800	2.03	25.49	17.39			0.619	0.01
TS + activin 8	TRIZOL	1716	2.05	28.16	17.95			0.642	0.01
TS + activin 9	TRIZOL	2030	2.03	25.00	17.40			0.524	0.01
TS + activin 10	TRIZOL	2099	2.03	24.00	17.81			0.602	0.01
TS + activin 11	TRIZOL	1868	2.04	25.83	17.76			0.589	0.01
TS + activin 12	TRIZOL	1811	2.04	25.27	17.67			0.560	0.01

Normal 1	TRIZOL	2327	2.00	25.81	17.33			0.961	0.05
Normal 2	TRIZOL	2372	2.00	27.01	17.04			0.986	0.05
Normal 3	TRIZOL	2431	2.00	25.98	17.32			0.950	0.05
Normal 4	TRIZOL	2254	2.01	26.96	17.49			1.181	0.05
Normal 5	TRIZOL	2194	2.02	26.18	17.53			1.099	0.05
Normal 6	TRIZOL	2782	1.98	26.48	16.94			0.905	0.05
Normal 7	TRIZOL	3100	1.93	33.40	16.77			1.105	0.05
Normal 8	TRIZOL	2664	1.98	28.23	17.21			1.085	0.05
Normal 9	TRIZOL	2922	1.96	25.97	17.20			1.123	0.05
Normal 10	TRIZOL	2527	2.00	26.00	17.55			1.134	0.05
Normal 11	TRIZOL	2400	2.00	26.00	17.77			0.928	0.05
Normal 12	TRIZOL	2314	2.01	27.00	17.73			0.5418	0.05
TS media only 1	TRYPLE	3889	1.7-1.95	19.74	17.07	0.0001	0.0000		
TS media only 2	TRYPLE	3935	1.7-1.95	20.01	17.57	0.0001	0.0000		
TS media only 3	TRYPLE	4075	1.7-1.95	25.7	17.21	0.0001	0.0000		
TS media only 4	TRYPLE	3148	1.7-1.95	22.47	17.17	0.0001	0.0000		
TS media only 5	TRYPLE	4058	1.7-1.95	26.63	16.42	0.0001	0.0000		
TS media only 6	TRYPLE	4180	1.7-1.95	27.35	16.52	0.0001	0.0000		
TS media only 7	TRYPLE	3666	1.7-1.95	27.07	16.65	0.0001	0.0000		
TS media only 8	TRYPLE	3782	1.7-1.95	27.42	16.52	0.0001	0.0000		
TS media only 9	TRYPLE	3596	1.7-1.95	27.41	16.62	0.0001	0.0000		
TS media only 10	TRYPLE	3305	1.7-1.95	27.55	16.75	0.0001	0.0000		
TS media only 11	TRYPLE	2940	1.7-1.95	28.02	17.44	0.0001	0.0000		
TS media only 12	TRYPLE	2970	1.7-1.95	28.18	18.32	0.0000	0.0000		
siRNA-337-1	TRYPLE	3084	1.7-1.95	25.69	17.08	0.158	0.006		
siRNA-337-2	TRYPLE	3407	1.7-1.95	26.36	16.82	0.180	0.006		
siRNA-337-3	TRYPLE	3511	1.7-1.95	31.65	16.65	0.200	0.006		
siRNA-337-4	TRYPLE	3467	1.7-1.95	26.89	17.22	0.188	0.006		
siRNA-337-5	TRYPLE	3093	1.7-1.95	25.86	17.29	0.157	0.006		
siRNA-337-6	TRYPLE	3727	1.7-1.95	29.48	16.19	0.176	0.006		
siRNA-337-7	TRYPLE	3572	1.7-1.95	26.66	16.56	0.137	0.006		
siRNA-337-8	TRYPLE	3374	1.7-1.95	30.31	16.82	0.154	0.006		
siRNA-337-9	TRYPLE	3687	1.7-1.95	28.12	16.19	0.177	0.006		
siRNA-337-10	TRYPLE	3815	1.7-1.95	29.19	16.38	0.195	0.006		
siRNA-337-11	TRYPLE	3880	1.7-1.95	28.9	16.39	0.190	0.006		
siRNA-337-12	TRYPLE	3747	1.7-1.95	31.02	16.32	0.213	0.006		
si negative control 1	TRYPLE	3370	1.7-1.95	31.09	16.56	1.093	0.028		
si negative control 2	TRYPLE	3541	1.7-1.95	30.98	16.75	1.216	0.028		
si negative control 3	TRYPLE	3815	1.7-1.95	30.43	16.56	1.012	0.028		
si negative control 4	TRYPLE	3688	1.7-1.95	30.36	16.79	0.922	0.028		
si negative control 5	TRYPLE	3911	1.7-1.95	29.76	16.29	0.945	0.028		
si negative control 6	TRYPLE	3408	1.7-1.95	29.01	16.64	0.838	0.028		

si negative control 7	TRYPLE	3425	1.7-1.95	30.29	16.46	0.903	0.028
si negative control 8	TRYPLE	3274	1.7-1.95	31.52	16.9	1.057	0.028
si negative control 9	TRYPLE	3549	1.7-1.95	29.13	16.56	0.969	0.028
si negative control 10	TRYPLE	3660	1.7-1.95	29.91	16.43	1.018	0.028
si negative control 11	TRYPLE	2323	1.7-1.95	25.94	17.73	1.001	0.028
si negative control 12	TRYPLE	3478	1.7-1.95	27.39	16.97	1.026	0.028
siRNA-733 1	TRIZOL	2687		26.62	16.46	0.40	0.01
siRNA-733 2	TRIZOL	2345	2.03	31.79	16.88	0.38	0.01
siRNA-733 3	TRIZOL	2480	2.02	27.85	17.06	0.35	0.01
siRNA-733 4	TRIZOL	2409	2.03	29.66	16.81	0.35	0.01
siRNA-733 5	TRIZOL	2058	2.04	28.01	16.98	0.36	0.01
siRNA-733 6	TRIZOL	2172	2.04	28.08	17.07	0.36	0.01
siRNA-733 7	TRIZOL	2180	2.03	32.17	17.21	0.35	0.01
siRNA-733 8	TRIZOL	2277	2.03	32.70	17.03	0.34	0.01
siRNA-733 9	TRIZOL	2095	2.05	35.23	16.95	0.38	0.01
siRNA-733 10	TRIZOL	2219	2.03	29.77	17.36	0.42	0.01
siRNA-733 11	TRIZOL	2260	2.04	26.80	17.20	0.35	0.01
siRNA-733 12	TRIZOL	1756	2.04	>35	17.52	0.39	0.01