The Role of Elf5 in Mouse Trophoblast Stem Cells

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Abstract

Elf5 is a DNA transcription factor that has been identified as being involved in placentation of the early embryo. Elf5 is expressed in the extra embryonic ectoderm (ExE), a lineage that contributes to the placenta of the embryo. Pluripotent trophoblast stem (TS) cells can be derived from the ExE and can be cultured *in vitro*. Such cells can contribute to all the placental lineages when injected back into an embryo. Elf5 homozygous mutant embryos do not possess an ExE and trophoblast stem cells cannot be derived and the mutation is therefore embryonic lethal. When Elf5 is knocked out, the TS cells in the ExE are thought to differentiate into EPC/giant cells leading to the absence of the ExE. In previous experiments in this laboratory, potential Elf5 targets were identified by RNA interference in mouse trophoblast stem cells followed by global gene expression analysis using an Affymetrix array. In the present experiments these results have now been confirmed by siRNA induced knockdown of Elf5 in TS cells followed by quantitative real time PCR of potential target genes. Most of the target genes that were affected by Elf5 knockdown were changed in the same way by growth factor removal and therefore stem cell differentiation. This suggested that Elf5 usually acts to maintain the TS cells in a stem cell fate. As Elf5 is expressed in the ExE, whole mount *In situ* hybridisation was used to determine if the genes showed the correct spatial and temporal patterning to be *in vivo* relevant Elf5 targets. The targets that were down regulated upon *Elf5* knockdown (and therefore positively regulated by Elf5) were expressed in the ExE as expected. Genes that were up-regulated upon *Elf5* knockdown (and therefore usually repressed by Elf5) were expressed in the differentiated ectoplacental cone or giant cells. . Preliminary work was also carried out for the over-expression of Elf5 by a tamoxifen inducible Elf5. TS cells were stably transfected with a plasmid containing *Elf5* fused to a tamoxifen inducible ERT2 receptor and a VP16 transcriptional activation domain to turn Elf5 into a potent activator, and were analysed for target gene expression. Preliminary data showed that Elf5 is involved in a complex transcriptional network of events as the genes did not behave as expected when Elf5 was turned into an activator. The effect of *Elf5* knockdown on TS cells has been studied closely in terms of

morphology, proliferation, changes in DNA content and apoptosis. The knockdown of *Elf5* caused an increase in the number of differentiated cells (as shown by changes in morphology and DNA content). This further supported the knockdown and *in situ* data. There was no change in proliferation or apoptosis. These experiments which demonstrate which genes are regulated by Elf5 and the changes to TS cellular characteristics when *Elf5* is knocked down, support the proposal that Elf5 acts as a trophoblast stem cell maintenance factor of the extraembryonic ectoderm of the mouse.

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CMV	Cytomegalovirus
cDNA	copy DNA
CM	conditioned media
Ct	threshold/crossing point
DMSO	Dimethyl sulphoxide
EPC	Ectoplacental cone
ES	Embryonic Stem
ExE	Extra embryonic ectoderm
FgF4	Fibroblast growth factor 4
FACS	Fluorescent activated cell sorting
Geomean	The geometric mean of three housekeeping genes
ICM	Inner cell mass
MEF	mouse embryonic fibroblast
miRNA	micro RNA
mRNA	messenger RNA
mTS media	mouse trophoblast stem cell media
OHT	4-hydroxytamoxifen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qRT-PCR	quantitative real time polymerase chain reaction
RNAi	RNA interference
RT	Room temperature
siRNA	small interfering RNA
TE	trophectoderm
TS	trophoblast stem
Tx	4-hydroxytamoxifen

1 Chapter 1: Literature Review

1.1 Overview

The purpose of this research is to delineate the molecular cascade underlying placental development. The placenta is the first organ to develop (Simmons and Cross, 2005) and is vital for embryonic development as it mediates the interaction between the growing foetus and the maternal blood stream (Rossant and Cross, 2001). At the early blastocyst stage of embryo development, two cell lineages can be distinguished, the inner cell mass (ICM) and the trophectoderm (TE). The ICM gives rise to the embryo proper and the extra embryonic endoderm, the TE to the foetal part of the placenta. The polar trophectoderm which lies above the ICM receives signals from the ICM to form the extra embryonic ectoderm (ExE) and the ectoplacental cone (EPC) (Simmons and Cross, 2005). The EPC and ExE contribute to the placenta which is made up of a mixture of foetal and maternal compartments. Pluripotent trophoblast stem (TS) cells can be derived from the ExE and can be cultured *in vitro* in the presence of embryonic conditioned media, FGF4 and Heparin (Tanaka et al., 1998). A DNA transcription factor, Elf5 has been identified that is essential for placental development. Elf5 is expressed in the ExE lineage and hypothesised to be required for TS cell maintenance (Donnison et al., 2005). *Elf5* homozygous mutants die around day 8.5-9.5 due to a failure to form an ExE. TS cells cannot be derived from these embryos suggesting Elf5 is essential for the preservation of TS cells *in vivo* (Donnison et al., 2005). To investigate why Elf5 is essential for the trophoblast lineage, Deane (2007) identified Elf5 target genes by RNA interference in TS cell lines, followed by global gene expression analysis using an microarray(affymetrix). This gave a group of genes that are up or down regulated upon loss of *Elf5*. The identification of genes that are regulated by Elf5 provided insight to its role in the placenta.

1.2 Early Development

Pre-implantation development begins with fertilisation and finishes with implantation which occurs at day 4.5 of development in the mouse. Following fertilisation the embryo undergoes a few rounds of cleavage with compactation occurring at the 8 cell stage, this is termed the morula. All the cells of the early morula are identical in terms of their morphology and development potential. During compactation the cells of the morula polarize and make epithelial like contacts. Calcium and the membrane binding protein E-cadherin is required during compactation and is involved in cell polarisation and formation of cell to cell contacts (Kunath et al., 2004). Successive radial or tangential cell divisions occur to give outer polar cells (radial/symmetric division) or inner apolar cells (tangential/asymmetric division). If left undisturbed the outer polar cells go on to give rise to the trophectoderm (TE) while the inner cells give rise to the inner cell mass (ICM) (Johnson and Ziomek, 1983; Kunath et al., 2004), see Figure 1 and Figure 2. Up to the 16 cell stage these lineage decisions are not irreversible as embryos assembled solely from inner or outer cells can develop into normally structured blastocysts (Suwinska et al., 2008). At the 32 cell stage only the inner cells have this same developmental potential. When 16 or 32 cell embryos are disaggregated the majority of the cells return to their original position within 24 hours. This indicates positional factors are important during this stage of development (Suwinska et al., 2008). Once the late embryo reaches the blastocyst stage at day 3.5 the lineage decisions are made and this flexibility is no longer seen (Kunath et al., 2004).

The trophoblast lineage provides the main components of the placenta whereas the ICM gives rise to the embryo proper as well as, the extra embryonic membranes such as the allantois and the amnion (Rossant, 2001). This lineage segregation is determined by the expression of key transcription factors, Oct4 (octamer-binding transcription factor 4) and Cdx2 (caudal type homeobox protein 2) (Niwa et al., 2005).

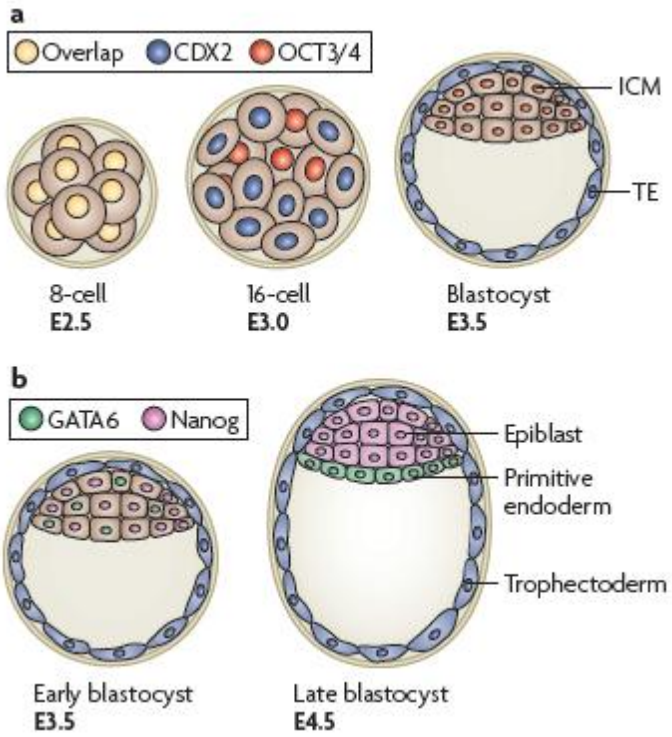


Figure 1: Expression of the key genes in early development (Arnold and Robertson, 2009).

Oct4 is initially expressed in all cells of the early embryo but then becomes restricted to the ICM of the blastocyst by Cdx2 (Figure 1) (Ralston and Rossant, 2008). Oct4 is a pluripotency marker of the ICM. *Oct4* homozygous mutant embryos can develop to the blastocyst stage but the ICM cells are not specified properly and the cells express trophectoderm markers (Nichols et al., 1998). Mouse embryonic stem (ES) cells can be derived from wild type ICM and epiblast. They can be maintained *in vitro* in the presence of leukaemia inhibitory factor (Williams et al., 1988). ES cells express *Oct4*, and when differentiation is induced *Oct4* is down regulated (Rossant, 2001). ES cells cannot be derived from *Oct4* mutant embryos and when *Oct4* is repressed in ES cells they differentiate into trophoblast giant cells (Niwa et al., 2000). This data indicates Oct4 is essential for lineage allocation in the early mouse embryo and is required for the self renewal of ES cells.

The HMG box transcription factor Sox2 plays a similar role to Oct4 as it preserves the ICM development potential, but unlike Oct4 it is also required in the proliferating trophoblast (Rossant, 2001). Sox2 is thought to play a role in

regulating Fgf4 expression. The down regulation of *Sox2* in both ES and TS cells causes differentiation into other cell types (Avilion et al., 2003).

The homeobox gene *Nanog* is expressed in the ICM and is also required for ES cell self renewal and maintenance of ICM cell fate. In *Nanog* mutants, ES cells and ICM cells differentiate into extraembryonic endoderm rather than extraembryonic ectoderm, which occurs when *Oct4* is deleted (Strumpf et al., 2005). This Suggests Oct4 and Nanog regulate different pathways in early development. The outer layer of the ICM forms the primitive endoderm (see Figure 1 and Figure 2); the primitive endoderm contributes to the yolk sac of the embryo. Expression studies and lineage tracing have shown that the random and mosaic 'salt and pepper' nature of expression of *Nanog* and *Gata6* determine what cells form the epiblast and the primitive endoderm respectively (see Figure 1). At E3.5 ICM cells exclusively express *Nanog* or *Gata6* and this determines the fate of the cell (Arnold and Robertson, 2009).

The ICM also expresses two other signalling molecules Nodal and Bone morphogenic protein 4 (Bmp4). *Nodal* is expressed in the epiblast and visceral endoderm and is involved in anterior-posterior patterning of the embryo. Nodal induces Bmp4 in the ExE (Beck et al., 2002). The role of Bmp4 in the ICM is unclear as *Bmp4* knockout embryos can develop normally up to gastrulation. Bmp4 is also necessary for the formation of germ layers and primordial germ cells in the ExE (Pfister et al., 2007).

Cdx2 is initially expressed in all cells of the early morula but then becomes restricted to the outer cells of trophectoderm lineage at the 16 cell stage (E3.0) (see Figure 1) (Niwa et al., 2005). Cdx2 is a key transcription factor involved in the trophoblast lineage decision. *Cdx2* mutants form normal blastocysts but they fail to maintain trophectoderm, instead forming a sphere of *Oct4* positive cells (Kunath et al., 2004). Cdx2 is required for the repression of Oct4 and Nanog in the trophectoderm (Ralston and Rossant, 2008; Strumpf et al., 2005). The restriction of Oct4 by Cdx2 results in the first lineage specification of the blastocyst therefore trophectoderm is the first lineage to be specified (Rossant

and Tam, 2009). *Cdx2* over-expression is also capable of inducing trophoblast differentiation in mouse embryonic stem (ES) cells (Niwa et al., 2005). As *Cdx2* mutants are capable of forming blastocysts another transcription factor may act up-steam of Cdx2 to initiate trophectoderm formation. The TEA-domain family member 4 (Tead4) has recently been identified and is required for trophectoderm lineage specification (Nishioka et al., 2008). *Tead4* mutants have a more severe phenotype than *Cdx2* mutants and *Cdx2* expression cannot be maintained in these embryos indicating its expression is required before Cdx2 (Yagi et al., 2007).

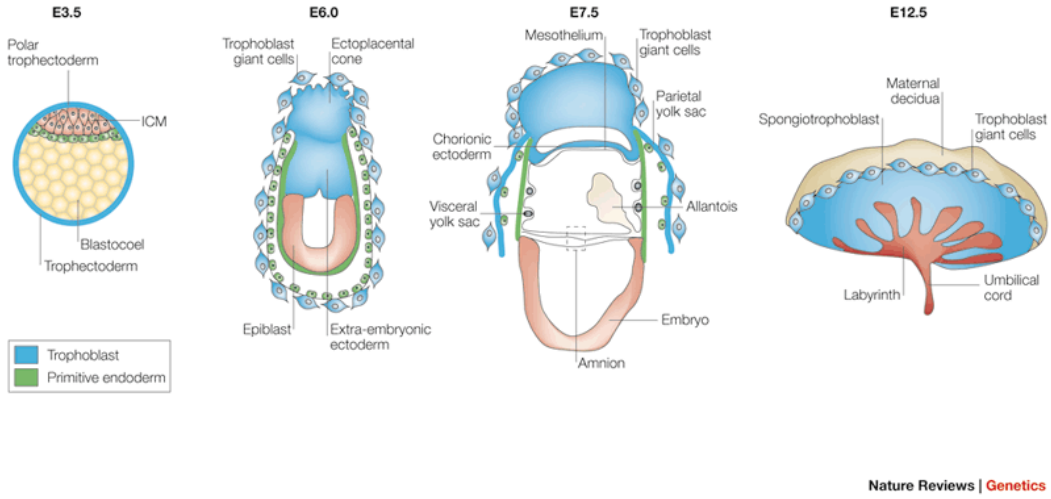


Figure 2: Placental development in the mouse. The blastocyst forms at E3.5 from there the polar trophectoderm forms the ExE and the EPC while the ICM forms the epiblast and the extra embryonic membranes. Far right are the components of the placenta (Rossant and Cross, 2001).

Once the first lineage decision is made the trophectoderm cells overlying the inner cell mass, known as the polar trophectoderm continue to proliferate and differentiate before implantation from day 4.5 to form the extraembryonic ectoderm (ExE) and the ectoplacental cone (EPC) (see Figure 2) (Gardner et al., 1973). The cells away from the ICM, the mural trophectoderm stop dividing but maintain DNA replication (endoreplication) to give rise to primary trophoblast giant cells which make contact with the maternal epithelium during implantation (Rossant, 2001; Rossant and Cross, 2001). The maintenance of the proliferation of the ExE is due to signalling from the ICM. In the absence of these signals the default pathway of giant cell differentiation occurs (Gardner et al., 1973). Fgf4 signalling is a major pathway involved in the continued proliferation and the

prevention of differentiation of the trophoblast. The proliferative potential of the ExE is sustained by a population of trophoblast stem (TS) cells (Tanaka et al., 1998).

1.3 Placental Development and Gene Expression

The extra-embryonic ectoderm is formed from the polar trophectoderm at day 4.5-5.0, and gives rise to the chorion at day 7.5. The ExE and the chorion express the marker genes *Cdx2, Eomes* and *Errβ*. These genes are involved in the trophectoderm lineage decisions and trophoblast maintenance.

The allantois which arises from the mesoderm at the posterior end of the embryo makes contact with the chorion. This event is termed chorioallantoic fusion. The allantois gives rise to the umbilical cord which is vital for nutrient and waste exchange of the embryo. During chorioallantoic fusion folds appear in the chorion which mark the sites where blood vessels will form (Rossant and Cross, 2001). From here the labyrinth will form the inner part of the placenta at day 8.5 (see Figure 2 and Figure 3). The labyrinth contains branched vili and a large surface area that is responsible for nutrient, gas and waste exchange between the developing foetus and the mother (Simmons and Cross, 2005). The placenta is a vital organ for the developing embryo as it also provides blood flow and produces growth factors and hormones (Cross et al., 2003).

The labyrinth consists of different types of cells and is thought to arise from the chorion. The cell types include mononuclear trophoblast cells that express placental lactogen II, syncytiotrophoblast cells and foetal endothelial cells (Simmons and Cross, 2005). The syncytiotrophoblast cells of the labyrinth start to develop at E8.5 when the labyrinth is formed. Syncytiotrophoblast cells express the transcription factor *GCM1*; which is required for their differentiation. Syncytiotrophoblast cells arise via cell cycle exit following by the fusion of two cells to give a multinucleated cell containing two diploid nuclei (Cross et al., 2003). Mouse TS cells can also give rise to syncytiotrophoblast cells when they are induced to differentiate *in vitro.* The lineage allocation of the placenta was

determined primarily by the expression of marker genes, see Figure 4(Cross et al., 2003).

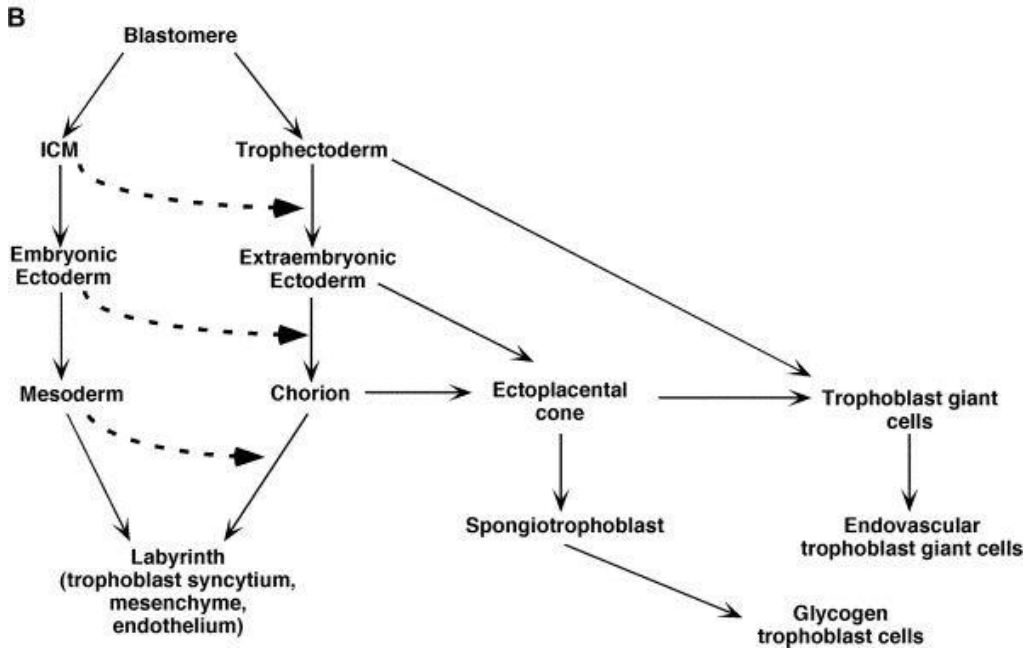


Figure 3: Diagram representing the trophectoderm lineages in the mouse which form the functioning placenta (Cross, 2005).

The ectoplacental cone (EPC) is also derived from the polar trophectoderm at day 4.5-5.0 (see Figure 2). As gestation continues the outer part of the ectoplacental cone gives rise to secondary giant cells which are in direct contact with the maternal decidua. Trophoblast giant cells are in contact with the maternal tissue at every stage during development and they mediate the invasion of the placenta into the maternal decidua. Trophoblast giant cells produce factors that promote blood flow to the site of implantation and are associated with maternal arteries (Simmons and Cross, 2005). The rest of the ectoplacental cone forms the spongiotrophoblast which lies between the labyrinth and the giant cells (see Figure 2). The function of the spongiotrophoblast is not well understood but it is thought to act as a support tissue for the developing villous structures of the labyrinth (Simmons and Cross, 2005). At around day 12.5 glycogen trophoblast cells form from the spongiotrophoblast and migrate towards the decidua and invade the uterus. See figure three for a diagram of the different lineages of the placenta.

The cell lineage decisions are controlled by the expression of different genes and transcription factors (see Figure 4). Genes involved in EPC and spongiotrophoblast allocation include *Tpbp* and *Flt1*. They are first detectable in the EPC and then later in the spongiotrophoblast. These genes are not expressed in trophoblast giant cells, the ExE or the labyrinth (Cross et al., 2003). The basic helix-loop-helix transcription factor Mash2 is expressed in the chorion, EPC and the spongiotrophoblast. Mash2 seems to prevent trophoblast differentiation into trophoblast giant cells and instead promotes EPC and spongiotrophoblast formation as loss of *Mash2* results in the loss of the spongiotrophoblast and allows giant cell differentiation to occur (Cross, 2005). In contrast to Mash2, the bHLH transcription factor Hand1 promotes giant cell formation (Simmons and Cross, 2005); these two transcription factors have opposing functions.

Isolated ExE or TS cells can give rise to giant cells *in vitro* in the absence of FgF4. They must go through a phase where they first express EPC and spongiotrophoblast markers *Mash2* and *Tpbpa* (Simmons and Cross, 2005). Giant cells are characteristic of the mouse and rodent placenta and endoreplication of these cells can give a DNA content of up to 1024N (Zybina and Zybina, 1996). Giant cells first arise when the mural trophectoderm is formed. Secondary giant cells arise when the some of the EPC cells differentiate into giant cells (Cross et al., 2003).

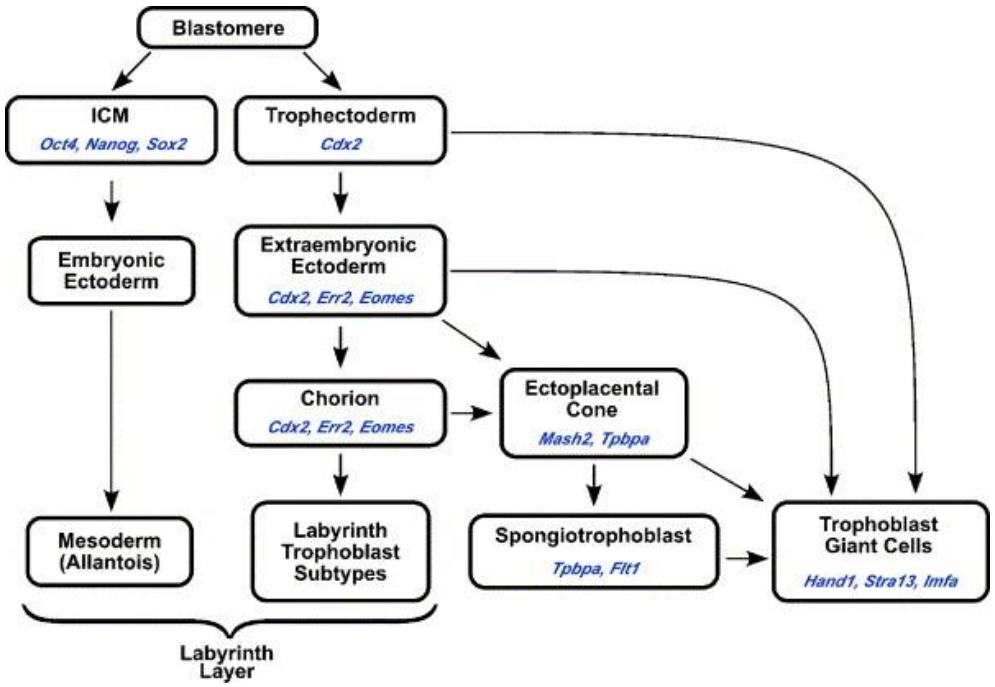


Figure 4: Gene expression of key genes in different lineages of the mouse placenta (Simmons and Cross, 2005).

1.4 Trophoblast Stem Cells as a Model for Placental Development

The proliferative potential of the extra embryonic ectoderm is dependent on a population of trophoblast stem cells which reside there**.** Signals from the ICM are thought to maintain the pluripotency of the TS cells (Simmons and Cross, 2005). A major signalling molecule involved in the maintenance of TS cell pluripotency is FGF. *Fgf4* is expressed in the early embryo, becoming restricted to the ICM of the blastocyst and later the epiblast (Niswander and Martin, 1992; Rappolee et al., 1994). The FGF4 receptor *Fgfr2* is expressed in the ExE, indicating FGF4 signalling from the epiblast to the ExE may be involved in trophoblast proliferation via this receptor (Simmons and Cross, 2005). Embryos with mutations of either *Fgf4* or *Fgfr2* are very similar and they fail to maintain trophoblast proliferation and identity (Arman et al., 1998; Feldman et al., 1995; Goldin and Papaioannou, 2003). Embryonic stem cells can be derived from these embryos but TS cells cannot, indicating FGF signalling is required for TS cell maintenance (Wilder et al., 1997).

Trophoblast stem (TS) cells have been isolated from the blastocyst at D3.5, and the ExE at D6.5 (Tanaka et al., 1998) and the choronic ectoderm (Uy et al., 2002). Tanaka et al. (1998) isolated and cultured TS cells from the ExE of D6.5 embryos on a feeder layer of primary mouse embryonic fibroblast (EMFI) cells in the presence of FGF4 and heparin. The removal of FGF4, heparin or EMFI cells resulted in a rapid decline in proliferation and differentiation into giant cells. TS cells can be cultured in the absence of feeder cells by using 70 % embryonic conditioned media supplemented with Fgf4 and heparin (Tanaka et al., 1998). TS cells can be injected back into blastocysts to create chimeras and the TS cells can only contribute to the trophoblast lineage of chimeras (Tanaka et al., 1998). Trophoblast stem cells can give rise to all of the differentiated trophoblast subtypes *in vivo* and *in vitro*.

Embryonic fibroblast cells secrete additional factors that help to maintain TS cells in a proliferative, undifferentiated state. Transforming growth factor β (TGF- β) and/or activin are key components of the embryonic fibroblast conditioned media (Erlebacher et al., 2004; Guzman-Ayala et al., 2004). Members of the TGF-β superfamily have been shown to be involved in the maintenance of TS cells. Members of this family include Nodal, BMPs and Activins. These molecules signal through type I and II transmembrane serine/threonine kinase receptors to their downstream Smad proteins. Activin and Nodal share the same receptors while TGF-β signals through Tgf receptors. Activin, Nodal and TGF-β activate the Smad pathway by causing the phosphorylation of smad2/3 which complexes with smad4 and translocates to the nucleus to activate transcription (Natale et al., 2009). Although these proteins signal through the same pathway they have different roles in the cell.

During gastrulation, Nodal is involved in antero-posterior patterning and is expressed in the epiblast and visceral endoderm. The proteases Furin and Pace4 are expressed in the ExE and are required to activate the Nodal precursor (Beck et al., 2002). Furin, Pace4 and Nodal are required to maintain TS cells in the ExE during gastrulation. Nodal sustains FGF4 expression in the epiblast and acts directly on the ExE in conjunction with FGF4 to maintain the expression of TS cell

marker genes (Guzman-Ayala et al., 2004). Although Nodal has been shown to be important *in vivo* for TS cells it can be replaced by activin *in vitro* for TS cell maintenance (Natale et al., 2009).

TS cells can be grown in the absence of embryonic fibroblast conditioned media by replacing it with Activin or TGFβ, while still supplementing with Fgf4 (Erlebacher et al., 2004; Natale et al., 2009). Erlebacher et al. (2004) was able to maintain TS cells in an undifferentiated state in media supplemented with Activin for 1.5 months after which these cells could still contribute to all placental subtypes in chimeras. The combination of Fgf4 and Activin or TGFβ can act together to maintain the expression of TS cell marker genes and prevent their differentiation into giant cells (Simmons and Cross, 2005). Although both Activin and TGF-β can both act to maintain TS cells they have different effects on the TS cells upon the withdrawal of Fgf4. Activin promotes the differentiation of labyrinth trophoblast subtypes such as syncytiotrophoblast cells. Trophoblast giant cells produce Activin and may act in a negative feedback loop to promote the formation of other cell types in the absence of FgF4 (Natale et al., 2009). In contrast to Activin, TGF-β alone promotes the formation of trophoblast giant cells (Natale et al., 2009).

1.5 Gene Expression in Mouse Trophoblast Stem Cells

Over 100 genes are known to be required for placental development (Cross, 2005) and are expressed at different stages in development. Markers of the extra embryonic ectoderm are also expressed in trophoblast stem (TS) cells. Markers include *Errβ, Cdx2, Fgfr2* and *Eomes*. These genes are down-regulated when differentiation is induced (Tanaka et al., 1998). Cdx2 (caudal –type homeobox 2) and Eomes (eomesodermin homologue) belong to transcription factor gene families and are downstream targets of FGF signalling (Rossant and Cross, 2001). Both are expressed in the ExE and are required for early trophoblast development. Cdx2 is an early marker for the undifferentiated trophectoderm lineage and is involved in the trophectoderm or ICM choice at the morula stage (Niwa et al., 2005; Ralston and Rossant, 2008; Strumpf et al., 2005). Eomes is required later than Cdx2, at the polar trophectoderm/mural trophectoderm

choice at the blastocyst stage (Donnison et al., 2005; Russ et al., 2000). Eomes is required later than Cdx2 as *Eomes* mutants do not show reduced *Cdx2* levels, while *Cdx2* mutants do show reduced *Eomes* levels (Rossant and Tam, 2009). *Eomes* mutants arrest in development at the blastocyst stage as the trophectoderm cannot form trophoblast and TS cells are not maintained (Russ et al., 2000). *Eomes* mutants fail to implant. The orphan nuclear receptor Errβ is also expressed in the ExE and the chorionic ectoderm. Unlike Cdx2 and Eomes, Errβ is required for trophoblast maintenance and not the lineage decision. *Errβ* mutants die much later, around D10.5 due to an arrest in trophoblast development (Rielland et al., 2008). Another transcription factor that has been recently discovered that is involved in early lineage allocation is Tead4. *Tead4* is expressed from the 2 cell stage with a maximum expression at the 8 cell stage. *Tead4* mutants die before implantation, do not express *Cdx2* and TS cells cannot be derived. Conversely ICM specific genes are still expressed and ES cells can be derived (Yagi et al., 2007). If *Tead4* is knocked out after implantation embryos can complete development. This indicates Tead4 is now on top of the transcriptional hierarchy involved in the development of the trophectoderm.

1.6 Elf5 in the Early Placenta

Elf5, an epithelial specific transcription factor of the ETS super family is involved in maintaining the extra-embryonic ectoderm lineage of the placenta (Donnison et al., 2005). The ETS family of transcription factors share a highly conserved DNA binding domain and recognise and bind to a purine-rich GGA(A/T) core at promoters and enhancers of target genes (Zhou et al., 1998). Elf5 was initially found to be expressed in the mammary gland and other epithelial tissues and in the neonatal mouse and during embryogenesis (Zhou et al., 1998). Zhou et al., (1998) also revealed that Elf5 is expressed in the placenta in mice from day 9.5 onwards.

To study the function of Elf5 during early embryogenesis, two groups produced a mutated non-functional *Elf5* allele using ES cells (Donnison et al., 2005; Zhou et al., 2005). Zhou achieved this by replacing exon 3 with a NLS-LacZ-neo construct and Donnison by inserting the puromycin gene into exon 2 of the *Elf5* gene.

Heterozygote *Elf5* +/- mice were viable and fertile. *Elf5* +/- mice were crossed but no *Elf5* homozygous mutants were obtained by either group indicating embryonic lethality (Donnison et al., 2005; Zhou et al., 2005). Normal Mendelain ratios were seen up to E6.5 indicating embryo lethality occurs sometime after gastrulation.

Zhou et al., (2005) observed that *Elf5* +/- females gave birth to viable offspring but their litters frequently died 24 to 48 hr after birth. This was found to be due to the inability of the female mice to lactate. Upon further investigation it was revealed that proliferation and differentiation in the mammary gland was defective and the secretory initiation phase was also inhibited (Zhou et al., 2005). Therefore Elf5 is essential for mammary gland development during pregnancy and one allele is not adequate for mammary gland function.

Donnison et al,. (2005) studied the role of Elf5 in early embryogenesis. *In situ* hybridisation revealed *Elf5* is expressed before gastrulation in the ExE lineage and is maintained during gastrulation. It was expressed at D5.5 to D8.5 with low levels detected by reverse transcriptase PCR (RT-PCR) at D3.5. *Elf5* was not expressed in the EPC (Donnison et al., 2005).

Interestingly *Elf5* homozygous mutants do not form an ExE. Whole mount *in situ* hybridisation of *Elf5* knockouts revealed the ExE molecular markers *Cdx2, Eomes, Fgfr2, Furin* and *Spc4* were not expressed at E6.5-7.5 confirming the absence of the ExE. The *Elf5* mutants could still form an EPC which suggests undifferentiated TS cells may not be maintained leading to an absence of the extra embryonic ectoderm. In confirmation of this, TS cells could not be isolated from *Elf5* homozygous mutant embryos. They were still able to be isolated from *Elf5* heterozygote embryos.

Elf5 mutant embryos were smaller than their littermates and exhibited no signs of primitive streak formation. *Elf5* null embryos also show patterning defects in the epiblast, this was shown to be due to defects in mesoderm formation

(Donnison et al., 2005). The mutant embryos did not express *Nodal* or any of its down-stream markers which are required for primitive streak formation.

The authors suggested Elf5 is a marker for undifferentiated TS cells, as in *Elf5* homozygous mutant embryos TS cells differentiate into EPC progenitors causing an absence of the ExE and embryonic lethality (see Figure 5). The absence of the ExE may also cause the defects in the embryo patterning as Nodal activity is dependent on the ExE to provide the proteases Furin and Spc4 to cleave the precursor protein into its active form. From all this data it was proposed that Elf5 acts downstream of Cdx2 and Eomes and is required for the decision between the ExE and the EPC which occurs after the blastocyst has implanted (see Figure 5). As the EPC is present in homozygous mutants this means the polar trophectoderm has formed normally allowing the differentiation into the EPC. Eomes is required for the formation of the pTE and therefore is not affected at this stage by the removal of *Elf5*.

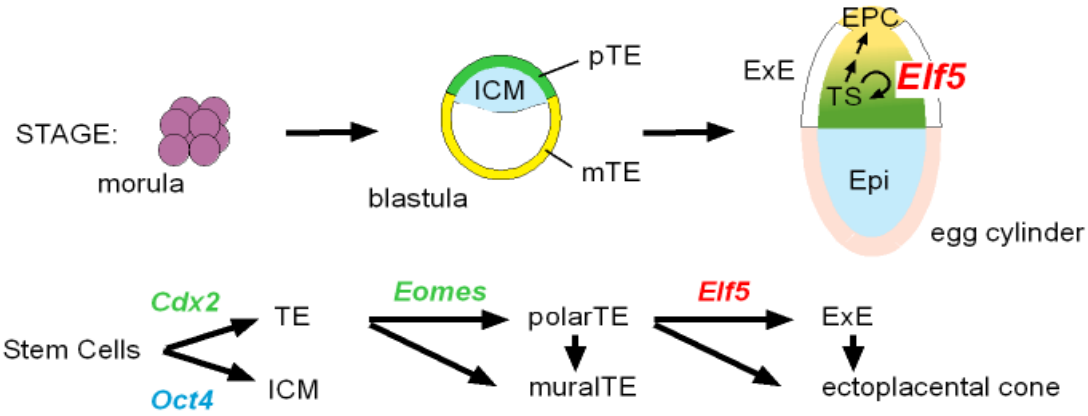


Figure 5: The role of Elf5 in the formation of the mouse embryo. It is required after the formation of the polar trophectoderm, at the choice between ExE and EPC. In the absence of Elf5 TS cells differentiate into EPC. Figure provided by Peter Pfeffer, AgResearch, Ruakura.

Recently Ng et al., (2008) showed that *Elf5* expression was repressed in ES cells by methylation. In TS cells *Elf5* was shown to be hypomethylated and therefore expressed. *Elf5* can be expressed in ES cells via a mutation in the DNA methyltransferase *Dnmt-1*. They also find that *Elf5* is expressed in the ExE and its expression is initiated later than Cdx2, which confirms the results by Donnison et al. The over expression of *Elf5* in ES cells causes differentiation of the ES cells into

trophoblast cells and an up regulation of trophoblast cell markers like Cdx2 and Eomes. Interestingly they find that the over expression of *Elf5* eventually causes the terminal differentiation into trophoblast giant cells.

Another ETS family of transcription factor member, Ets2 is also expressed in the trophoblast from E5.0-6.75 and its phenotype is similar to Elf5. *Ets2* mutants show a decrease in growth of the EPC, and some mutants lack ExE (Wen et al., 2007) and its molecular markers (Georgiades and Rossant, 2006). The absence of *Ets2* in the trophoblast (and therefore absence of the ExE) prevents primitive streak and mesoderm formation and improper localisation of the AVE. (Georgiades and Rossant, 2006). The inactivation of *Ets2* also results in the prevention of TS cell self renewal. *Ets2* inactivation causes the down regulation of *Cdx2, Errβ* and *Pace4*, but interestingly not *Furin* (Wen et al., 2007). The lack of signalling from the ExE probably causes the defects seen in embryo patterning (Georgiades and Rossant, 2006).

Deane (2007) identified putative target genes of Elf5 by loss of *Elf5* function via small interfering RNA (siRNA) expression in TS cells. This was followed by an Affymetrix array and quantitative real time PCR (qRT-PCR) to give a group of genes that are up or down regulated upon the loss of *Elf5*. These target genes are potentially involved in TS cell function and further studies can give insight to the pathways involved in trophoblast development. One such target is *Sox2*, *Sox2* knockouts die around implantation as it is essential for ICM development and later in the ExE and TS cells, it is thought to be a pluripotency marker (Avilion et al., 2003).

1.7 Purpose of This Study

The purpose of this study was to delineate Elf5 target genes and thus ascertain the role of Elf5 in placental development. Putative Elf5 target genes have been identified by Deane (2007), some of the most interesting targets were studied by repeat knockdown in mouse trophoblast stem cells. To further verify the genes that are regulated by Elf5, Elf5 was over-expressed by using a tamoxifen inducible Elf5. The expression of the proposed Elf5 targets was examined *in vivo*

to determine if they displayed the correct spatial and temporal pattering to be a target of Elf5. Once *Elf5* was knocked down, the TS cells were characterised in terms of morphology, DNA content, proliferation and apoptosis to see if there was any change compared to untreated cells, to determine if cells with *Elf5* knocked down were still in fact TS cells. The identification of Elf5 targets and their function gave insight into TS cell maintenance and placental development.

2 Chapter Two: Methods

All experimental work was carried out at AgResearch Ruakura, Hamilton. For the preparation of media, reagents and buffers, refer to the appendices.

2.1 Cell Culture Protocols

All general Trophoblast stem cell and mouse embryonic fibroblast cell culture protocols were obtained from the book 'Placenta and Trophoblast, methods and protocols, volume 1, chapter 11 (Quinn et al., 2006). Cell culture was carried out in a laminar flow hood (Westinghouse Pty Ltd, NSW, Australia) which contains a 0.2 µm HEPA filter and blows sterile air outwards to keep the contents of the hood sterile. The laminar flow hood was routinely wiped with 70 % ethanol before and after use and was kept going at all times, everything entering the hood was also thoroughly wiped with 70 % ethanol. Cells were grown and maintained in a 37 °C/ 5% CO_2 water jacket incubator (Forma Scientific).

2.1.1 Mouse Trophoblast Stem Cell Culture

Mouse trophoblast stem m(TS) cells were maintained in media containing 70 % embryonic fibroblast conditioned media containing 25 ng/ml FGF4 (Sigma-Aldrich) and 1 µg/ml Heparin (Sigma-Aldrich) (growth factors). Conditioned media provides additional growth factors that keep the mTS cells in a proliferative undifferentiated state with a tight epithelial morphology (Tanaka et al., 1998). The media was routinely changed every 48 hours. The pH of the media was monitored by the colour of the phenol red in the media. All media was pH adjusted to pH 7.2-7.4 with 1M HCl before filter sterilising through a 0.22 µM filter.

2.1.1.1 Passaging of mTS Cells

Cells were passaged every 48-72 hr using TrypLE™ Express (Gibco, Invitrogen). The frequency of passaging depended on the initial seeding density, which was usually 1:5 to 1:20 of the original culture. When the cells reached 80-90 % confluency they were passaged into a new well or plate to prevent

differentiation and to allow continued growth. For normal culture the cells were grown in 6-well dishes. For general passaging, the media was removed from cells and they were washed gently with PBS. 300 µl TrypLE Express was added to the cells to dissociate them and they were incubated for 4 min at 37 °C. After 4 min the cells started to form singular rounded cells as observed by using an inverted phase contrast Olympus CK2 microscope. When the cells were rounded and were beginning to detach 1 ml of mTS media was added to dilute the TrypLE Express. The media was pipetted up and down to remove the adherent cells and then centrifuged at 1000 rpm for 5 min in a Mistral 1000 bench top centrifuge (Labsupply, Auckland, New Zealand). The supernatant was aspirated and the cells were re-suspended in 1ml mTS media. For passaging every second day the cells were re-plated at a density of 1:10 of the original seeding density.

2.1.1.2 Counting of Mammalian Cells

To count TS or MEF cells, the cells were lifted from the culture plate by TrypLE Express as in section 2.1.1.1 centrifuged in a MSE Mistral 1000 centrifuge and re-suspended in 1 ml of mTS media (TS cells) or DMEM (MEFs), refer to appendices for preparation of the media. 10 µl of the cell suspension was then counted on a Neubauer haemocytometer using a 20 x inverted phase contrast Olympus CK2 microscope. The depth of the chamber was 0.1 mm and the central area 1 mm^2; therefore the volume was 0.1 mm^3 or 1 x 10^{-4} ml. The number of cells in each of the 4 of the 4 x 4 squares was counted and then divided by 4. To calculate the number of cells per ml the number of cells counted was multiplied by volume so n x 10^4 would give the number of cells per ml.

2.1.1.3 Thawing of Cells

Stocks of surplus cells were kept in liquid nitrogen (-196 °C). When the cells were removed from long term storage they were rapidly thawed by placing the cryovial in a 37 °C water bath until just thawed. Care was taken to prevent immersion in the water bath for too long as the cells become susceptible to the DMSO in the freezing media. Once thawed the vial was sprayed thoroughly with 70 % ethanol and the cells were placed in 5 ml of fresh mTS media and centrifuged for 5 min at 200 x g. The supernatant was removed and the cells

were re-suspended in 2 ml of 70 % conditioned media containing growth factors. The cells were usually maintained in one well of a 6 well plate. The cells were passaged 2-3 times after thawing before use for experiments as the cells can take a while to recover from the freezing process.

2.1.1.4 Freezing of mTS Cells

When the cells were in excess they were frozen down to replenish the liquid nitrogen stocks. 1 x 10^6 cells were centrifuged for 5 min at 200 x g, the supernatant was aspirated and the cells were re-suspended in 1 ml of mTS media. 1 ml of freezing media was added to the cell suspension, which contained 500 µl FCS, 300 µl mTS and 200 µl DMSO. The cells were divided into two cryovials and put into a "Mr Frosty" (Nalgene™ Cryo 1 °C Freezing container) containing isopropanol and frozen at -80 °C, this allowed slow freezing of the cells to prevent ice crystal formation which can cause damage to the cells. The following day the vials were transferred to liquid nitrogen for long term storage.

2.1.2 Mouse Embryonic Fibroblasts

Mouse embryonic fibroblast (MEF) cells were used to produce the growth factors required for trophoblast stem cell maintenance. Without these growth factors trophoblast cells would differentiate into giant cells (Tanaka et al., 1998). The MEFs were kept at a low passage number (between passages 1-10) so they could continue to produce high levels of growth factors. MEFs were mitomycin C treated so the cells could produce growth factors without using them up themselves for growth and metabolism. Mitomycin C inhibits DNA synthesis by binding to DNA which induces DNA cross-linking

2.1.2.1 Preparation of MEFs

The MEFs were grown up in DMEM media (refer to appendices) before mitomycin C treatment. Upon thawing MEFs were seeded into a T25 flask (Nunc), and once confluent they were spilt 1:6 into 6 T75 flasks (Nunc). The cells were washed once with PBS, then 1 ml of TrypLE Express was added to the flask and the cells were left at 37 °C until the cells became rounded and began to

dislodge. The TrypLE was then neutralised with DMEM and the cells were centrifuged down for 5 min at 1000 rpm. The supernatant was removed and 6 ml of fresh DMEM was added to resuspend the cells. The cell suspension was spilt into 6 flasks containing 25-30 ml of DMEM. The cells were left for about one week until they became confluent. Once the cells were confluent, one flask was kept for re-seeding into 6 new flasks and the other 5 flasks were mitomycin C treated.

2.1.2.2 Mitomycin C Treatment

Mitomycin C prevents the replication of the MEFs so they produce growth factors but are not dividing so they don't need to use the growth factors themselves. The majority of the media in the flasks to be mitomycin C treated was removed, leaving 5 ml in the bottom. 50 µl of a 1 mg/ml solution of mitomycin C (Sigma-Aldrich) was added to the flask to give a final concentration of 10 µg/ml of mitomycin C. The flasks were returned to the incubator and left for four hours at 37 °C. After this time the cells were washed three times with PBS and were then trypsinised. Once the cells were dislodged they were centrifuged and the supernatant was removed. The cells were resuspended in 5 ml of mTS media and 10 µl was counted using a haemocytometer. The cells were then divided into flasks to give 2 x 10^5 cells per ml of mTS media. On average one confluent flask would provide 25-30 ml of conditioned media. The media in each flask was removed every 72 hours, three times. After the media was removed for the third time the cells were discarded. The conditioned media produced, contained the growth factors required for the maintenance of the trophoblast cells in a stem cell fate. The media was centrifuged, the supernatant was removed and filter sterilized through a 0.22 µm filter stericup (Nalgene Nunc International, USA) using a vacuum pump and then frozen at -20 °C until use.

2.1.2.3 Culture of mTS Cells in Activin

Activin can be used in place of conditioned medium (Erlebacher et al., 2004). Due to this we changed to using 10 ng/ml Activin plus 25 ng/ml of Fgf4 and 1 µg/ml Heparin in mTS media instead of using conditioned media. All other cell culture protocols were kept the same. The use of Activin was preferred as it is a more

controlled media, the amount of growth factors in the conditioned media can change with every batch.

2.1.3 Transfection of cells with RNA -Transient Transfection

2.1.3.1 Seeding of the Wells

For a general transfection TS cells were grown up in one well of a 6 well plate. When the cells were about 80-90 % confluent the cells were passaged as in section 2.1.1.1 and resuspended in 1 ml of mTS medium. As a general rule about 50-100 µl of this cell suspension was then added to each well of a 6 well plate for a 48 hr long transfection to prevent the overgrowth of the colonies. The cells were seeded at a slightly higher density for a 24 hr long transfection. The cells were grown in 70 % conditioned media with Fgf4 and heparin or activin until it was time to transfect the cells. The cells were about 30 % confluent at the time of transfection the following day.

2.1.3.2 Transfection Procedure

Transfections were carried out in 12 well or 6 well dishes. The Elf5 siRNA and negative control siRNA was obtained from invitrogen. All transfections contained a scrambled si negative control that was run alongside each transfection to account for any off target effects. The concentration used in 12 well plates was 50 nM of siRNA (invitrogen) and 4 µl of lipofectamine 2000 (invitrogen). In 6 well plates it was 50 nM siRNA plus 10 µl of lipofectamine 2000.

2.1.3.3 Transfection in a 6 Well Dish:

100 pmol siRNA (5 µl of a 20 pmol solution) was added to 250 µl OptiMEM reduced serum medium (Gibco) at the same time 10 µl lipofectamine 2000 was added to 250 µl OptiMEM in a separate tube. These mixtures were incubated at room temperature (RT) for 5 min. After the 5 min incubation the two mixtures were combined and incubated at RT for 20 min. During this incubation time the media was replaced with 1.5 ml reduced serum (5 % FCS) mTS media as serum can reduce the transfection efficiency. After 20 min incubation the siRNA-

lipofectamine mixture was added to the cells drop wise to create a final volume of 2 ml.

2.1.3.4 Transfection in a 12 well dish:

40 pmol siRNA (2 µl of a 20 pmol solution) was added to 98 µl of OptiMEM. At the same time 4 µl of lipofectamine was added to 96 µl of OptiMEM. These mixtures were incubated alone as above for 5 min and then combined and incubated for a further 20 min at room temperature. During this incubation time the media was replaced with 600 µl reduced serum (5 % FCS) mTS media. After 20 min incubation the siRNA-lipofectamine mixture was added to the cells drop wise to create a final volume of 800 µl.

The cells were incubated for 4-6 hours in the presence of the RNA. After this time the cells were rinsed with PBS and fresh complete media was added to all cells. The cells were then incubated for the required time period (24 or 48 hr). When the time period was reached the cells are washed with PBS and the cells were either fixed or if the RNA was to be isolated, the appropriate amount of Trizol Reagent (invitrogen) was added. For 6 well plates 500 µl of Trizol reagent was added to each well. For a 12 well plate 200 µl of Trizol was added.

2.1.4 Stable Transfection of TS cells

TS cells were stably transfected with the Elf5-mo-Vp16-ER-pyCAGizeo plasmid. This vector was for the over-expression of the Elf5 protein. To further validate the Elf5 targets a tamoxifen inducible Elf5 was used. The opposite trends should be observed when Elf5 is over expressed. Tamoxifen binds to the estrogen receptor ligand binding domain of the Elf5-ER fusion protein causing translocation into the nucleus and the inducible activation of Elf5 targets. The VP16 transcriptional activation domain has been added to ensure robust transcription.

2.1.4.1 Preparation of Plasmid DNA

Before transfection into the TS cells the plasmid must be linearised first. 4 µg of DNA was required for one transfection and the transfection was carried out in quintuplets so 20 µg of DNA was required. 30 µg of DNA was digested with a restriction enzyme which cut the vector in the ampicillin resistance gene and then it was purified using a Wizard column see section 2.3.2.4.1.

2.1.4.2 Preparation of TS Cells

This protocol was obtained and modified from Himeno et al.,(2008).

To obtain an adequate number of cells for a stable transfection, the TS cells were grown up in 100 mm culture dishes. When the cells were about 80 % confluent the media was removed, they were washed with PBS and 1 ml of TrypLE Express was added. The cells were then put back in the incubator at 37°C for 4 min to lift the cells. 5 ml of mTS media was then added and gently pipetted up and down to break the cell aggregates. The cells were then washed and resuspended in 6 ml of mTS media. The cells were then counted and diluted in TS medium to give a cell number of 5×10^5 cells/ml.

2.1.4.3 Preparation of the Lipofectamine Complex

4 µg of the plasmid DNA was diluted in 250 µl of OptiMEM reduced serum media in a 1.5 ml eppendorf tube. In another tube 10 µl of lipofectamine 2000 was mixed with 240 µl of OptiMEM and incubated at room temperature for 5 min. After the 5 min incubation the DNA and lipofectamine mixtures were combined and incubated at room temperature for 20 – 40 min.

2.1.4.4 Transfection of the TS Cells

As the cells were not already plated at the time of transfection, this class of transfection is described as a reverse transfection. The DNA/lipofectamine complex was added to a 35 mm petri dish (non tissue culture, but sterile) and then 1 ml of the cell suspension was added and mixed by gentle pipetting. For a control cell line 1 ml of cells were mixed with 500 µl of 70 % conditioned media. The cell/lipofectamine complex was then returned to the incubator and left for 4-5 hr.

After 4-5 hr the cells were transferred to a larger dish to allow growth of the cells during antibiotic selection without the need for passaging. The un-adherent cells were removed and placed in a 15 ml falcon tube. The adherent cells were washed with PBS and the PBS was transferred to a falcon tube (in case any cells come off during the wash) and then 300 µl of TrypLE Express was added to the cells and incubated for a few minutes until the remaining cells became loose. The cells were re-suspended in mTS media, centrifuged and then re-suspended in 10 ml of 70 % conditioned media with Fgf4 and heparin. The cells were then plated in a 100 mm tissue culture dish.

2.1.4.5 Antibiotic Selection for the Introduced Plasmid

As the introduced Elf5-mo-VP16-ER-pyCAGizeo plasmid contained the gene for zeocin resistance the cells containing the plasmid integrated into their genome could be selected for with zeocin (invitrogen). 24 hours after the transfection the media was replaced with media containing 200 µg/ml zeocin. Zeocin causes cell death by intercalating into DNA and cleaving it. Zeocin resistance is obtained by the *Sh ble* gene product which inactivates zeocin by binding to it to prevent it binding to DNA (Invitrogen, 2009). The media was changed every second day until the cells without the plasmid began to die. As zeocin is quite a slow acting antibiotic this could take up to 2 weeks. The rate of cell death could be monitored by the control cell line. The control cell line was not transfected so when these cells died off the remaining cells could be considered as Zeocin resistant and therefore contained the plasmid DNA integrated into their genome.

2.1.4.5.1 Modified Protocol- Selecting With Puromycin

As zeocin is a slow acting antibiotic a rapidly acting antibiotic was used in following transfections. Puromycin is a fast acting antibiotic and cell death should occur within a couple of days. Puromycin resistance is conferred by expression of the *Pac* gene and must be introduced on a separate plasmid. A molar ratio of 1/10 of the Elf5-mo-Vp16-ER-pyCAGizeo plasmid was required as it is not optimal to have lots of colonies that are resistant to puromycin but do not contain the plasmid of interest. Cells were transfected as per the protocol outlined above in

section 2.1.4.2 with both linearised plasmids. Selection with 1 µg/ml of puromycin (Sigma-Aldrich) began 24 hr after the beginning of the transfection.

2.1.4.6 Picking Stably Transfected Lines

Once the non transfected cells had died off Zeocin or puromycin resistant colonies began to appear. These colonies were picked to establish stably transformed clonal cell lines. Before the colonies were picked sufficient 24-well plates were prepared containing 10 µl of PBS (enough for the number of colonies to be picked). The media was removed from 100 mm dishes and replaced with 15 ml of PBS. A TS cell colony was picked with 5-10 µl PBS using a 20 µl pipette under a microscope in the laminar flow hood. The colony was transferred to a well of the 24 well plate. After all the colonies were picked from one 100 mm dish 50 µl of TrypLE Express was added to each well containing a colony and incubated at 37 °C for 4 min. Each colony was pipetted gently to break up the colonies and 200 µl of 70 % conditioned media containing 1.5x the standard concentration of growth factors was added to each well. The same procedure was repeated for the other 100 mm dishes. The plates were incubated for 24 hr at 37 °C and then the media was replaced with 0.5 ml of 70 % CM with Fgf4 and heparin and 20 µg/ml zeocin or 1 µg/ml puromycin.

2.1.4.7 Screening for Insertion into Genomic DNA

To ensure the plasmid containing the Efl5-VP16-ER fusion was inserted into the genome, the genomic DNA was isolated from the clonal cell lines and a PCR was carried out to amplify a region of the plasmid. During passaging 100-200 µl of the cell suspension was put aside for genomic DNA isolation. The cells were centrifuged at 2,000 rpm for 10 min and the supernatant was removed. The cell pellet was re-suspended in 5-10 µl of lysis buffer containing 1mg/ml of proteinase K (Roche, Germany) and incubated at 55 °C for 1 hr. 45-90 µl of MQ water was added to the samples, mixed and then incubated at 100 °C for 5 min. The genomic DNA samples were stored in the fridge until required for the PCR. 0.5-0.05 µl of purified genomic DNA was used in the PCR reaction to amplify a region on the plasmid, see section 2.3.1.8 for the PCR protocol.

2.1.4.8 Over Expression of Elf5

The over expression of Elf5 was induced upon the addition of 4-hydroxytamoxifen (OHT) where the fusion protein can translocate into the nucleus and fulfil its transcriptional functions. The cell lines for analysis were plated out the day before in 12 or 6-well plates to give an optimal density of 30-50 % confluence the day of induction. The following day the cells were treated with 0, 0.5 µg/ml and 1 µg/ml 4-hydroxytamoxifen (Sigma), returned to the incubator and left for 24 hr in the dark (OHT is light sensitive). After this time the RNA was isolated for analysis by real time PCR (see section 2.3.1.1 to section 2.3.1.7).

2.1.4.9 Immunocytochemistry

Immunocytochemistry was carried out on fixed cells to ensure the cells were expressing the Elf5-VP16-ER protein and that it translocated into the nucleus upon the addition of 4-hydroxytamoxifen (OHT).

2.1.4.10 Fixation

Cells were plated into 8-well chamber sides and left for 24 hr. After 24 hr 1 µg/ml of OHT was added to the induced well and left in the incubator in the dark for another 24 hr. Following the 24 hr incubation the slides were washed twice with PBS and then fixed with either 4 % paraformaldehyde (PFA) at room temperature for 5 min or with 100 % methanol at -20 °C for 10 min. The slides were then washed with PBS twice and covered with PBS and left at 4 °C until use.

2.1.4.11 Permeabilisation

If the cells were fixed with methanol permeabilisation was not required as methanol produces holes in the cell membrane. If they were fixed with PFA the cells were permeabilised with 0.1 % Tritron-X-100 for 5 min at room temperature and then washed twice with Tris-buffered saline (TBS).

2.1.4.12 Staining

Non specific binding was prevented by blocking with 1 % goat serum for 30 min at room temperature. The slides were then washed three times with TBS-tween.

The primary antibody was diluted in TBS-tween containing 0.1 % BSA and incubated for an hour at room temperature. The unbound antibody was washed off by washing with large volumes of TBS-tween three times for 10 minutes each while rocking. The secondary antibody was diluted in TBS-tween with 0.1 % BSA and was left on the cells in the dark for one hour at RT. Unbound secondary antibody was washed off with TBS-tween three times 10 min in the dark. The slides were then washed with 1:10,000 dilution of H33342 (invitrogen) for 10 min, then washed twice with distilled water and left to drip dry. The chambers were removed and the slides were mounted with a cover slip containing fluorescent mounting media. The slides were left to dry in the dark at RT over night. The next day the slides were analysed for fluorescence in the blue (H33342) and green spectrum (alexa 488 secondary antibody). An Olympus BX50 microscope was used to observe the fluorescent cells.

2.2 Cell Analysis Protocols

2.2.1 Morphology

2.2.1.1 Preparation of the Cells

The morphology of TS cells was analysed by staining with Phalloidin-TRITC (Sigma) and Hoechst 33342 (invitrogen). The protocol was obtained from the Sigma product information sheet.

This procedure was carried out to determine the morphological changes of the TS cells when *Elf5* is knocked down. *Elf5* was knocked down as in 2.1.3.3. As no changes were observed at 2 days we decided to investigate changes in morphology 5-6 days after *Elf5* knockdown. Due to this long period the cells were passaged 48 hr after transfection to prevent the cells becoming too confluent. The cells were trypsinised, 1 ml of mTS media was added to lift the cells then the cells were pipetted up and down. As giant cells tend to adhere to the culture dishes a lot stronger than TS cells the remaining cells were scraped off. The cells were spun down, re-suspended in 1 ml and then 100 µl of this suspension was added to each well of a 2-well chamber slide. The cells were then grown for a further 3-4 days, with the media being changed every two days.

2.2.1.2 Fixation and Permeabilisation of the Cells

When the cells were ready for morphological analysis the media was removed and the cells were washed twice gently with PBS. The cells were then fixed with 300 µl of 4 % paraformaldehyde for 5 min at RT. The PFA was then removed by aspiration and the cells were washed three times with PBS. The cells were then permeabilised with 300 µl 0.1% Triton x-100 for 5 min at room temperature. The Triton X-100 was removed by aspiration and the cells were washed again with PBS twice.

2.2.1.3 Staining of the TS Cells

The cells were stained with 5-20 µg/ml phalloidin for 30 min at room temperature in the dark. Once the actin was stained the unbound phalloidin was removed by washing three times with PBS. Following this 20 µg/ml H33342 was added to the chamber and left for 5min at RT. The cells were washed once again with PBS. The PBS was removed, the chambers removed and a few drops of fluorescent mounting medium was added to each slide. The slide was then covered with a cover slip and left over night in the dark at room temperature to dry. The following day the slides were put in the fridge until photos were taken. Photographs were taken using an Olympus BX50 fluorescent microscope.

2.2.1.4 Flow Cytometry

For flow-cytometric analysis, a FACScan™ (Beckton Dickinson, San Jose, CA, USA), with an Argon laser (488 nm) was used. The machine was calibrated every time before use with CaliBRITE™3 beads (BD Biosciences) to ensure there was sufficient separation for FSC, SSC, FL1-FL3 channels using the FACSComp software (BD). The beads were to adjust instrument settings, set fluorescent compensation and to check instrument sensitivity. The forward and side scatter settings were adjusted so the majority of the events fitted inside a line FSC/SSC dotplot. A total of 10,000 events were acquired for each measurement. The data was evaluated using Cell Quest Pro software (BD). Robin McDonald provided assistance every time optimisation was required for a new assay.

2.2.2 DNA Content

To confirm the morphology results, the DNA content of the TS cells was determined upon *Elf5* knockdown. Propidium iodide (PI) can be used to stain DNA; the amount of PI in the cell is proportional to the amount of DNA in a cell. Flow cytometry was used to determine the DNA content of the cells. PI can be used for cell cycle analysis and determination of the ploidy of a cell. PI was useful here because when the TS cells differentiate into giant cells their DNA content increases due to endoreplication (refer to section 1.3). So the proportion of giant cells in a cell population of a given treatment can be determined by measuring the DNA content. This protocol was provided by Robin McDonald. This is a standard PI staining procedure.

Elf5 was knocked down as in 2.2.1.1 and the cells were passaged once as above. After 5-6 days the cells were trypsinised, PBS was added to neutralise the trypLE and then pipetted up and down and the remaining cells were scraped off. The TS cells that had been in media with no growth factors for 6 days had to be trypsinised for longer as giant cells are more resistant to TrypLE Express.

2.2.2.1 Modification to Protocol

The cells tended to be quite clumpy due to scraping the remaining giant cells. So instead of scraping the cells that had de-attached after 4-5 min of TrypLE Express treatment were removed and another 300 µl of TrypLE Express was added to each well. The cells were incubated at 37 °C until the remaining cells came off. It was very important that a single cell suspension was obtained as the cells can only pass one at a time through the sample injection port of the FACS to get an accurate reading.

2.2.2.2 Fixation

The cells were centrifuged, PBS removed and the cells were re-suspended in 100 µl of PBS. 900 µl of ice cold 70 % ethanol was added to fix the cells. The cells were stored at -20°C for at least 24 hr. The following day the cells were counted and 1-2x10^6 cells were aliquoted into tubes.

2.2.2.3 Labelling of DNA

The samples were centrifuged for 3 min at 300 x g to remove the ethanol. The cells were washed twice by centrifugation and were re-suspended in 500 µl PBS. To this 500 µl of DNA extraction buffer was added and the cells were vortexed gently to suspend the cells. They were then incubated at RT for 10 min. The DNA extraction buffer was removed by centrifugation and the cells were re-suspended in 1 ml DNA staining buffer containing 50 µg/ml PI (invitrogen) and 50 µg/ml RNase A (invitrogen). The cells were vortexed briefly and incubated in the dark for at least 30 min at room temperature. The cell suspension was passed through BD filter tops to remove any cell clumps before analysis by flow cytometry. DNA QC particles (BD) were used for verification of instrument performance and quality control of the FACS before DNA analysis. These particles ensured there was a good separation between the G1, S, and the G2/M phases of the cell cycle. DNA staining by PI was measured in channel 3 (FL3, bandpass > 600 nm) with linear amplification.

2.2.3 Proliferation

Proliferation assays were used to determine if there was a change in proliferation when *Elf5* was knocked down. The protocol was obtained from the cell signalling website from invitrogen. This was a general protocol for antibody labelling of cells for flow cytometry.

2.2.3.1 Controls

Cell cycle-dependent H3 phosphorylation begins in late G2 and remains high through early anaphase (Hendzel et al., 1997). As a positive control, cells were treated with 15-100 ng/ml Nocodazole for 16 hr. Nocodazole halts cells in the G2 phase and therefore can be used as a positive control for the phosphorylation of histone 3 at serine 10 as this marker is at a maximum at G2/M phase of the cell cycle. Mitomycin C treatment was used as a negative control.

2.2.3.2 Knockdown and Fixation

Elf5 was knockdown as in section 2.2.1.1 and 2.2.2.1 and the cells were passaged once during treatment. The media was removed, and the supernatant was put into a 15 ml falcon tube. The cells were washed with PBS, and the supernatant was kept again as mitotic cells are loosely adhered to the plate. The cells were trypsinised as in 2.1.1.1, neutralised with PBS and all cells were removed all cells by pipetting up and down. The cells were centrifuged, the PBS aspirated and re-suspended in 1 ml PBS. 1 ml of 4 % PFA was added to each tube to give a final concentration of 2 % PFA. The cells were fixed for 10 min at 37 °C and then chilled on ice for a minute.

2.2.3.3 Permeabilisation

The cells were pelleted by centrifugation and then re-suspended in 5 ml of 90 % methanol and incubated on ice for 30 min. The cells could be stored in 90 % methanol at this step if required.

2.2.3.4 Staining Using Antibodies

The cells were counted and 1 x 10^6 cells/ml were aliquoted into tubes. 2-3 ml of incubation buffer (see appendices) was added to each tube, rinsed by centrifugation and then repeated. The cells were re-suspended in 100 µl incubation buffer and the cells were blocked at room temperature for 10 min. Blocking prevents non specific binding of the primary antibody. The primary antibody was added at a dilution of 1:100 to 1:500 of a 5 mcg/ml solution (had to be optimised) and incubated for 60 min at room temperature. The primary antibody was Anti-phospho-Histone H3 (Mitosis Marker). The Peptide corresponding to residues 7-20 of histone H3; recognizes Histone H3 phosphorylated on serine 10. This antibody reacts with all eukaryotes; the host was a rabbit (Upstate).

Following this the cells were rinsed twice in incubation buffer by centrifugation and the cells were resuspended in a 1:1000 dilution of an Alexafluor 488 goat anti rabbit IgG antibody (invitrogen). Incubation at room temperature for a further 30 min was carried out. The cells were rinsed once again in incubation

buffer and resuspended in 0.5 ml PBS. The cells were filtered through a 35 µm BD filter top to remove any clumped cells. The proliferation was then analysed on the flow cytometer. The measured events were gated on the FCS/SSC plot to exclude any sub cellular fragments and clumped cells. Alexa 488 staining was measured in channel 1 (FL1, bandpass 515-545).

2.2.4 Apoptosis

An Apo-BrdU Tunel Assay kit was purchased from Invitrogen and the protocol was followed and apoptotic cells were detected by flow cytometry. This protocol is also described by Darzynkiewicz et al., (2008). Positive and negative control cells were provided by the kit. The positive control was a Human Lymphoma cell line treated with camptothecin. The negative control was the same cell line but untreated. In every assay a negative control without the TdT enzyme was also included.

2.2.4.1 Cell Preparation and Fixation

Elf5 was knocked down according to previous protocols and apoptosis was analysed 48 hr and 5 days after knockdown. The media was removed, and the supernatant was kept as cells floating in the media are likely to be dead or apoptotic. The cells were washed with PBS, the supernatant was kept again. The cells were trypsinised as in 2.1.1.1 and 2.2.2.1, neutralised with PBS and all cells were removed by pipetting up and down. The cells were centrifuged, the PBS aspirated and the cells were resuspended in 0.5 ml PBS. 5 ml of 1 % PFA was added to the cell suspension and they were incubated on ice for 15 min. The cells were centrifuged for 5 min at 300 x g to remove the PFA and washed twice with 5 ml PBS. The cells were resuspended in 0.5 ml PBS and 5 ml of ice cold 70 % ethanol was added to the cell suspension. The cells were stored at -20 °C for at least 24 hr.

2.2.4.2 Detection of Apoptotic Cells

The cells were centrifuged for 5 min at 300 x g to remove the ethanol. The cells were washed twice with 1 ml of wash buffer. In the meantime 50 µl of the DNA

labelling solution was prepared for each treatment. The DNA labelling solution contained 10 µl of reaction buffer, 0.75 µl of TdT enzyme, 8 µl of BrdUTP and 31.25 µl of MQ water. A negative control was included at this step which was the absence of the TdT enzyme. The DNA labelling solution was added to the cells and they were incubated in a 37 °C water bath for 1 hour. The tubes were shaken every 15 min to keep the cells in suspension. After the 1 hr incubation the cells were washed twice by centrifugation with 1 ml of rinse buffer. The antibody staining solution was added to each sample which contained 5 µl of the Alexa Fluor 488 dye-labelled anti BrdU antibody and 95 µl of rinse buffer. The cells were incubated in the antibody solution for 30 min in the dark. After the 30 min incubation time 0.5 ml of propidium iodide/ RNase A DNA staining solution was added to each sample and they were incubated for a further 30 min in the dark. Before analysis on the flow cytometer the cells were passed through a 35 µm BD filter top to remove any clumped cells. The Alexa 488 signal was collected in channel 1 (FL1, bandpass 515-545). And the PI signal was collected in channel 3 (Fl3, bandpass > 600 nm).

2.3 Molecular Biology Techniques

2.3.1 Analysis of Gene Expression

2.3.1.1 RNA Isolation

RNA isolation was carried out in order to isolate the messenger RNA (mRNA) in the cell. mRNA is a RNA copy of the genes that have been transcribed and that will be then translated into protein. Therefore mRNA analysis is a suitable tool for gene expression analysis. The reactions were scaled up according to the amount of Trizol containing sample that was present to begin with. To 200 µl Trizol containing sample 1 µl pEnSpike and 1 µl MS2 RNA (800 µg/µl) was added. A spike was added to give an estimation of the yield of the RNA extraction at the end of the cDNA synthesis. MS2 RNA was used as a carrier. 40 µl chloroform was added and the samples were vortexed briefly, or shaken vigorously for 15 sec. The RNA containing samples were then centrifuged at 14,000 rpm for 15 min. The sample separated into three layers; the top aqueous phase contained the

RNA. The top aqueous phase was transferred into a new tube. An equal volume of isopropanol and 2 µl of linear acrylamide (5 µg/µl) were added to each sample. Linear acrylamide was added before precipitation to permit the visualisation of the RNA pellet. The samples were centrifuged again at 14,000 rpm at 4 °C for 30 min. The supernatant was removed and the pellet was washed with cold 70 % ethanol and centrifuged again for 10 min. The supernatant was removed and the RNA pellet was air dried, care was taken to not over dry the pellet. The RNA pellet was resuspended in 8 µl DEPC treated water.

2.3.1.2 DNase I Treatment

DNase I treatment was carried out on the isolated RNA to remove any genomic or contaminating DNA. It digests single and double stranded DNA to oligodeoxy-ribonucleotides containing a 5' phosphate. 1 µl of 10 x DNase I reaction buffer (Invitrogen) plus 1 µl DNase I (Invitrogen) was added to the RNA, and they were incubated at 37 °C for an hour. After this time 1 µl of 25 mM EDTA (Invitrogen) was added to each sample and was incubated at 75 °C for 10 min to inactivate the DNase. Following this 1.5 µl sodium acetate and 45 µl 100 % ethanol was added, this was left at -20 °C over night to precipitate the RNA. The following day the RNA was centrifuged at 14,000 rpm for 30 min, the RNA pellet was washed with cold 70 % ethanol and the pellet was air dried. The pellet was resuspended in 12 µl DEPC treated water.

2.3.1.3 cDNA Synthesis

cDNA synthesis involves making a single stranded copy of DNA from mRNA. The enzyme reverse transcriptase is an RNA dependent DNA polymerase that is isolated from a retrovirus. The reverse transcriptase enzyme needs a 3' primer as a start point; this is provided by the oligodT primer which hybridises with the polyA tail on the 3' tail of the mRNA. The enzyme can then synthesise a complementary DNA copy of the mRNA by incorporating dNTPs that are added in the reaction mixture. To 12 µl RNA sample, 1 µl of 10 mM dNTP mix (Roche, Germany) and 1 µl of an oligodT_{14}VN mix (invitrogen) was added to each sample. The sample was mixed and incubated at 65 °C for 5 min, then put on ice for a minute. 4 µl of a 5x first strand buffer (contains Mg2+ for the reverse

transcriptase, invitrogen) plus 1 µl of RNAse Out (an RNase inhibitor, invitrogen) and 1 µl of Superscript III reverse transcriptase (invitrogen) were added to each tube. The samples were mixed and incubated at 50 °C for 60 min to allow the enzyme to make a complimentary copy of the mRNA. After the 50 °C incubation, the temperature was raised to 70 °C for 15 minutes to inactivate the reaction and then put on ice to cool. 0.5 µl of RNase H (invitrogen) was added and the samples were placed at 37 °C for 30 min to remove the RNA of the RNA: DNA hybrid to leave a single stranded DNA copy of the mRNA message. After the RNase treatment 2 µl of sodium acetate and 60 µl of 100 % ethanol were added and the DNA samples were left at -20 °C overnight to precipitate. The following day the samples were centrifuged at 14,000 rpm for 30 min, washed with 70 % ethanol, air dried and the pellet was re-suspended in 100 µl T.1E. The DNA could then be amplified by real time PCR to see what genes were expressed and at what level.

2.3.1.4 Primer Design for Real Time PCR

The real time PCR primers were designed to amplify preferably the 3' region of the gene of interest. The cDNA sequence of the gene of interest was found by using the reference gene accession number or the name of the gene. The sequence was imported into vector NTi which has primer designing software. The primers were designed for real time PCR with specific requirements. The amplicon should be designed over an intron to ensure contaminating genomic DNA was not present. The product length was a minimum of 150 bp maximum 350 bp, Ta optimum of 56-60 °C, no complementary binding to avoid primer dimers. 18-22 nucleotides in length, avoid poly-base stretches to prevent inappropriate hybridisation, primers should have compatible melting temperatures (within 5 °C), 40-50 % GC, even spread of G and Cs and AA on the 3' end of both primers. With all these specifications the prefect primer pair was hard to come by therefore a few attempts may be required to get a good pair of primers to amplify the gene of interest. The primers were tested via reverse transcriptase PCR first to make sure one product was obtained. This was tested by running the product on an agarose gel and obtaining a single band. Also melt curve analysis was used make sure there was only one peak which will verify a

specific product. The PCR primers were resuspended in TE and then diluted to a 10 µM working stock in either DEPC treated water or T.1E.

2.3.1.5 Real Time PCR

Real time polymerase chain reaction (qRT-PCR) was used to amplify a gene of interest while simultaneously monitoring the amplification of the product in real time. Real time PCR was carried out in triplicate, where 2 µl of the cDNA was used in duplicates along with a 1:2 dilution in MQ water. A 1:2 dilution was used to ensure the amplification was in the linear phase of the reaction. The 1:2 dilution should be 1 cycle behind the neat sample. To 2 µl cDNA 5 µl of SYBR green master mix (Takara), 0.1 µl forward primer, 0.1 µl reverse primer and 2.8 µl of water was added to give a final volume of 10 µl. The product was then amplified in real time in the Corbett Research real time PCR machine. The cycling conditions that were used were as follows; hold at 95 °C for 3 minutes to denature the DNA, and then 40 cycles of 95 °C for 10 seconds, 60 °C for 15 seconds and then 60 °C for 20 seconds. After 40 cycles a melting step was performed from 72 °C to 99°C increasing by 1 °C in each step. If good results were not obtained with these conditions the annealing temperature was usually changed. If that was not successful GC rich solution was added to the master mix. And if that was unsuccessful the primers were redesigned.

2.3.1.6 Analysis of Results

The melt curve was analysed to ensure only one peak was present for each PCR primer pair. If there was more than one peak it was due to primer dimers or contaminating genomic DNA. When primers produce more than one peak the primers were usually redesigned. Once the melt curve had been analysed to ensure there was a clean melt curve, the comparative quantitation function was used to give the Ct value and the amplification efficiencies. The Ct values were analysed to ensure the duplicates were within ± 0.03 of each other and the 1:2 dilution was one cycle later ± 0.03. The Ct values and amplification efficiencies were then copied into an excel file with specific formulas to calculate copy number for each sample. The initial copy number was calculated by the equation: $N_o = 1/Ef^{Ct}$ (Wilkening and Bader, 2004).

Where N_o is copy number, Ef is efficiency and Ct is the crossing point or the Ct. The copy number was compared to the geometric mean of three housekeepers (Gapdh, β-actin and β-tubulin) to give relative expression levels. The change in gene expression was compared to an untreated sample as a control or a si negative control for the knockdown data.

2.3.1.7 Spike

Primers for the spike were used to amplify the spike RNA added to the RNA sample at the start of the RNA isolation. A good extraction usually yields a spike C_t value of about 20.0. If the C_t values were much later than this then it could be assumed that some RNA had been lost during the extraction. A spike was added to every sample during a RNA isolation.

2.3.1.8 Reverse Transcriptase PCR

Reverse transcriptase PCR was carried out to amplify a gene or DNA of interest. It was then followed by gel electrophoresis to examine expression of certain genes or to use the DNA for cloning. For a standard 25 µl reaction the following reagents were added to 1 µl cDNA synthesised in step 2.3.1.3. 19.8 µl MQ water, 2.5 µl 10x PCR reaction buffer (Roche), 0.5 ml 10 mM dNTP mix (Roche), 0.5 µl of each of the forward and reverse primers and 0.2 µl of the fast start Taq polymerase (Roche). A standard PCR would have the following cycling conditions; 95 °C for 5 min to denature the DNA, then 35-40 cycles of 95 °C for 30 sec to denature the DNA, 56-60 °C for annealing and 72 °C for 30 sec for elongation. Then a final elongation step at the end at 72 °C for 5-7 min. A PTC-200 Peltier Thermal Cycler was used for reverse transcriptase PCR. The amplified DNA was then stored at 4 to -20 °C until use.

2.3.1.9 Agarose Gel Electrophoresis

An agarose gel was used to view DNA fragments and to determine their molecular weight. DNA fragments can be separated according to size as smaller fragments run through the agarose gel faster than larger fragments. Depending on the size of the DNA fragment to be separated a 0.6-2 % gel was made. To separate small pieces of DNA a higher percentage gel was used as small

fragments travel through the gel matrix much easier than large fragments. To separate large fragments a low percentage agarose gel was used. Agarose powder was suspended in 0.1 % TAE buffer, heated in a microwave to dissolve the agarose and then poured in a gel cast with a comb and left to set. Ethidium bromide (Sigma) or SYBR safe (Molecular probes, invitrogen) was added to the gel before it was poured to allow visualisation of the DNA bands under UV light. A loading buffer was added to the DNA samples and then they were loaded into the wells of the gel. A molecular weight DNA ladder of 100 bp, 1 KB or an ultra ranger DNA ladder was added in an adjacent well at the same time so the size of the DNA bands could be determined. As DNA is negatively charged it would run towards the positive electrode when current was applied. The DNA separated according to size and then the samples with the correct size fragments were used for cloning or any other application.

2.3.2 Sub Cloning

Subcloning is the transfer of a gene of interest from one plasmid to another. Sub cloning was used to create the Elf5-mo-VP16-ER-pyCAGizeo plasmid. The Elf5-mo-ER-pyCAGizeo plasmid was provided by Craig Smith (a Scientist at AgResearch, Ruakura). The VP16 cassette was added as it is a transcriptional activation domain which may improve the transcription of the *Elf5* gene. The VP16 cassette was isolated from the pTet-Advanced plasmid (Clontech).

2.3.2.1 Restriction of the Vector

The vector into which the VP16 fragment was to be inserted into had to be restriction digested to open the vector at the site that the VP16 fragment was inserted into. In a 20 µl reaction 2-3 µg of the Elf5-mo-ER-pyCAGizeo plasmid DNA was cut to open with NheI for insertion of the VP16 product.

2.3.2.2 Dephosphorylation of the Vector

Dephosphorylation of the vector fragment ends was essential when the ends were compatible to each other. This prevented the re-ligation of the vector to itself. To 20 µl of the restricted plasmid DNA 24 µl of MQ water plus 5 µl of 10 X

CIP buffer (Roche) and 1 µl of CIP (Roche at 1 U/µl) was added. The mixture was incubated at 37 °C for 30 min then another 1 µl of the CIP was added and incubated for a further 30 min. The vector was then run on an agarose gel and gel purified (refer to gel purification 2.3.2.4).

2.3.2.3 Cloning using PCR

PCR was used to amplify the gene of interest for cloning. The Gene of interest was amplified for cloning by using primers containing a 5' restriction sequence of choice and a 3-4 bp clamp as enzymes do not cut very well at the end of fragments. Two primers with the restriction sites SpeI and NheI were used to amplify the VP16 cassette. 1 ng of plasmid DNA was amplified in a 50 µl PCR reaction. The cycling conditions used were 95 °C for 5 min then 30 cycles of 95 °C for 30 sec, 60 °C annealing for 30 sec and then 72 °C for 30 sec for extension. This was followed by 72 °C final extension for 10 min at the end. The PCR was carried out as a traditional PCR (see section 2.3.1.8) and then the fragment was restriction digested with SpeI and NheI to remove the clamp, gel purified and ligated into the vector

2.3.2.4 Gel Purification

DNA fragments were purified from an agarose gel when the fragments were needed for cloning. There are three methods that have been used here, the Wizard® (promega), GENECLEAN® (Obiogene), MERmaid®(BIO 101). These products can also be used to purify PCR products directly without running a gel.

2.3.2.4.1 Wizard

The Wizard system can be used to extract and purify DNA fragments of 100 bp to 10 Kb. The standard protocol is as follows. If purifying from an agarose gel, the gel slice containing the DNA of interest was weighed and 10 µl of membrane binding solution was added per 10 mg of gel slice. The mixture was vortexed and incubated at 50-65 °C until the gel slice dissolved. If beginning with a PCR product an equal volume of membrane binding solution was added to the PCR reaction. The dissolved mixture was added to the SV minicolumn which was placed in a collection tube. It was incubated at room temperature for one minute

and then centrifuged at 16,000 x g for one minute. The flow through was discarded and reinserted into the collection tube. The column was then washed twice with the membrane wash solution, the column was air dried and then the DNA eluted with 30- 50 µl of DEPC water or TE.

2.3.2.4.2 GENECLEAN

GENECLEAN can also be used to purify DNA from agarose gels or PCR reactions. The DNA bands were cut out of the gel, placed in pre weighed tubes and the gel slices were weighed. 4 volumes of Iodide mixture was added to each tube (kept at 4°C) and incubated at 55 °C for 5 min or until the gel was completely dissolved. The glass beads were vortexed vigoursly and then 5 µl of the glass beads were added to the mixture. The mixture was then centrifuged at full speed for one second. The supernatant was aspirated and 700 µl of cold ethanol wash was applied and the beads were resuspended. The sample was spun and washed again and the supernatant was aspirated. Then 5 µl of water was added to the purified DNA and incubated at 55 °C for 5 min, after this time the tube was spun again and the 5 µl of supernatant containing the purified DNA was retrieved.

2.3.2.4.3 MERmaid

This kit can also purify DNA from agarose gels or PCRs; only the gel purification method was used here. The MERmaid system can be used to successfully purify small fragments of DNA of 10-200 bp.

The glassfog was re-suspended and 400 µl was added to the spin filter. Up to 300 mg of gel slice was added to the matrix and it was heated in a 55 °C water bath to dissolve to gel. The sample was then incubated at room temperature for 5 min with agitation to mix and then centrifuged for 1 min at full speed to transfer the liquid to the catch tube. The matrix was then washed with 500 µl of the high salt wash and then washed twice with a cold ethanol wash. 10 – 25 µl of the elution solution was added to the filter and the glass fog was re-suspended by flicking the tube. The DNA was eluted by centrifugation.

2.3.2.5 Ligation

For cloning the VP16 DNA was either ligated into P-GEM Teasy for TA cloning or the *Elf5* tamoxifen inducible plasmid. Ligation was carried out using a Takara DNA ligation kit. To 2 µl DNA 1 µl of pGEM-T Easy plasmid and 3 µl of the Takara mighty mix was combined. For the VP16 cassette and the Elf5-mo-ER-pyCAGizeo plasmid, equivalent molar ratios were used. This was then incubated 30 min to over night at 16 °C. The mighty mix contains a T4 DNA ligase which joins the ends of the plasmid with that of the PCR product.

2.3.2.6 Transformation

The plasmid containing the PCR product was transformed into Invitrogen Max Efficiency® DH5α™ Competent Cells to amplify the plasmid containing the DNA of interest. Max Efficiency® DH5α™ Competent Cells were thawed on ice. The 1-2 µl of the ligation mix was added to the cells, and was mixed by stirring gently with a pipette tip. The cells were incubated on ice for 30 min. The cells were then heat shocked for 45 sec at 42 °C and then plunged into ice for 2 min. 200 µl SOC medium was then added and left shaking horizontally at 37 °C for an hour. During this time LB-Antibiotic plates were prepared containing 2.5 % LB broth, 1.2 % agar and 100 µg/ml Ampicillin. The vector contained ampicillin resistance so only colonies containing the vector would be able to grow (with or with-out an insert). After an hour the transformed bacteria were spread onto the plates and left agar-up at 37 °C over night. The following morning white colonies of *E.coli* could be seen growing on the plates. They were left in the fridge until ready for picking for the minipreps.

2.3.2.7 Minipreps

Minipreps were carried out to isolate the amplified plasmid from the bacterial culture. In the late afternoon white colonies were picked using sterile toothpicks and were put into 3 ml LB/Amp broth and incubated O/N shaking at 300 rpm at 37 °C. Up to 48 miniprep's were carried out at one time to try and isolate colonies with the correct insert. The following morning, 1.5 ml was poured into a 1.5 ml eppendorf tube for the miniprep and the other 1.5 ml was kept at 4 °C for a glycerol stock. The tubes were spun at 1100 rpm for 1 min, and the miniprep

procedure was followed. 100 µl solution 1 was added and the pellet was vortexed until it was fully re-suspended. Then 200 µl solution 2 (lysis buffer- 0.2 N NaOH, 1 % SDS) was added to each tube and mixed well by inversion. 150 µl of cold solution 3 was then applied and the tubes were again mixed well by inversion. The samples were centrifuged at 13,000 rpm for 5 min at 4 °C, the SDS precipitates and the supernatant containing the DNA was then transferred to a fresh tube. An equal volume of isopropanol was added and centrifuged again at 4 °C to precipitate the DNA. The supernatant was aspirated and washed with an equal volume of 70 % cold ethanol, centrifuged again and the DNA pellet was air dried briefly. The pellet was re-suspended in 30 µl TE containing 1:200 dilution of 10 mg/ml DF-RNAase A and incubated at 37 °C for 30 min. The purified DNA was stored at 4 °C until ready for restriction digestion.

2.3.2.8 Restriction Digest of DNA

To ensure the clones contained the correct insert the plasmid was restriction digested and then the products were run on an agarose gel to make sure they were the correct size. In a standard 20 µl reaction x mg DNA, 2 µl of restriction buffer and 1-2 µl of restriction enzyme was used. The volume was made up to 20 µl with MQ water. As a general rule no more than 1/10 of the volume should be enzyme as restriction enzymes are stored in a 50 % glycerol solution, high concentrations of glycerol can inhibit the restriction enzyme or cause non specific (Star) activity.

2.3.2.9 Maxi Preparation of DNA

A maxi prep was carried out to get enough DNA for sequencing and to transfect the cells with. 200 ml of LB broth was inoculated with 1 µl of an overnight culture kept in the fridge that was used for the minipreps. Only 1 µl was used to try and get a single colony with the fragment of interest in the plasmid. The Roche maxi prep procedure was followed. The culture was incubated overnight shaking at 300 rpm at 37 °C. The following morning the bacteria were centrifuged for 10 min at 5,000 x g at 4 °C. The supernatant discarded and the pellet was re-suspended in 12 ml of suspension buffer which contains RNase. The pellet was vortexed until the pellet was suspended in the buffer. 12 ml lysis buffer was

added to the suspension and mixed gently by inversion and then incubated on ice for 5 min. An SDS precipitate forms. The solution was separated from the precipitate by filtration. The column was then equilibrated and lysate was loaded on to the column and allowed to flow through (flow through discarded), the plasmid binds to the column. The column was washed two times with wash buffer. The column was put into a collection tube and the plasmid was eluted with 15 ml pre warmed elution buffer (50 °C). The DNA was then precipitated with 11 ml of isopropanol, mixed by inversion and centrifuged for 30 min at > 15, 000 x g at 4 °C. The supernatant was discarded and the pellet was washed with 5 ml chilled 70 % ethanol and centrifuged again. The ethanol was removed by the use of a vacuum pump. The pellet was re-suspended in 50 µl T.1E and left at 4 °C over night to dissolve. The next morning the DNA concentration was measured on the nanodrop. Other kits were also used (Invitrogen and Qiagen) but the general procedure was similar.

2.3.2.10 Sequencing

The maxiprep DNA was sent to the Waikato University DNA Sequencing facility to check the sequence of the ligation sites and the sequence of the insert.

2.3.2.11 Glycerol Stocks

Once a clone was obtained that had the VP16 inserted correctly a glycerol stock was made. 850 µl of the bacterial suspension saved from the miniprep was added to 150 µl of pure glycerol in a cryovial. The stocks were flask frozen by immersion in liquid nitrogen for a few seconds, and then stored at -80°C until required.

2.3.2.12 The Nanodrop

The nanodrop was used to estimate DNA or RNA concentration of a given sample. The machine was calibrated with water and then 'blanked' with whatever the DNA or RNA was re-suspended in (e.g. MQ or TE or T.1E). Then the concentration 1 µl of the sample was measured to give a concentration in ng/µl. The 260/280 and 230/280 absorbance ratios were also given which are an indication of solvent contamination and nucleic acid purity respectively. A

260/280 ratio of 1.8 or greater is considered pure for DNA; a lower ratio could be due to phenol, protein or other contaminants that absorb strongly at 280 nm. A 260/230 ratio measures nucleic acid purity, a pure sample has a ratio of 2.0-2.2.

2.3.3 Whole Mount *in situ* Hybridisation

2.3.3.1 Probe Preparation

Whole mount *in situ* hybridisation was used to examine the expression of the genes of interest in day 6.5-7.5 mouse embryos.

2.3.3.2 Primer Design

Primers were designed to amplify the 3' end of the gene of interest. The optimal length of the PCR product was 700-1000 base pairs. Highly conserved regions of the gene should be avoided so the probes do not hybridise with mRNA of other genes. The prevention of cross hybridisation can be ensured by using the Ensembl web site: http://www.ensembl.org/Mus_musculus/index.html and determining how many members are present in the superfamily. If there were many members in the superfamily a blast search was carried out http://blast.ncbi.nlm.nih.gov against the mouse genome. If the overall identity of the two genes was over 85 % that area that showed possible cross hybridisation was excluded and also short stretches with over 95 % identity were also to be excluded. If the region could not be excluded a note was taken in case of cross hybridisation later on. Primers were designed by using vector NTi software or by eye. The sequence of each gene was also checked for abnormally high or low GC content. As cloning was carried out by TA cloning, primers were designed with an A or a G at the 5' end as Taq polymerase is more likely to put an A on the 3' end of the product. Adding an A at the 5' end of the primer increases cloning efficiency (Peng et al., 2007).

Primers were ordered from Sigma and they were re-suspended in Tris-EDTA to give a 1 mM stock solution and were stored at -80 °C. Primers were then diluted 1:100 in DEPC treated water to give a 10 µM working solution, they are stored at -20 °C.

2.3.3.3 Cloning using PCR

The genes of interest were amplified using PCR. The PCR was carried out in a PTC-200 Peltier Thermal Cycler containing the following reaction mix;

MQ water 40.5 µl

10 x PCR buffer 5 µl

dNTP mix (10 mM) 1 µl

DNA template 1 µl

Forward primer (10 µM) 1 µl

Reverse primer 10 µM 1 µl

FastStart Taq Polymerase 0.5 µl

The 10x PCR buffer provides a stable chemical environment for the Taq polymerase including Mg^{2+}. The dNTP mix is deoxynucleotide triphosphates which were used as the building blocks for the new DNA. The samples were mixed and run at the following temperatures; 95 °C for 5 min (hot start), then 35 cycles of 94 °C 30 sec (denaturation), 60 °C 45 sec (annealing- primer dependant), 72 °C for 1 min (elongation). Then 72 °C for 10 min and then held at 4°C. Then 5 µl of the product was run on a 1 % agarose gel containing ethidium bromide with 1 µl gel loading buffer with a 100 bp ladder at 95 V until a good separation of the DNA bands was obtained.

2.3.3.4 Purification of the PCR Product

The PCR product had to be purified as Taq is difficult to inactivate and would fill in restricted ends, as primer dimers will compete for restriction enzymes and as nucleotides will interfere. Product purification was carried out using a Wizard® SV Gel and PCR Clean-Up System. The manufacturer's instructions were followed see 2.3.2.4.1 above. The eluted DNA was stored at -20 °C until further use.

2.3.3.5 Ligation of DNA into the Vector

As Taq DNA polymerase adds a 3' A to the end of the DNA strand (Zhou and Gomez-Sanchez, 2000) this single nucleotide overhang can be used in TA cloning

where the vector contains a single T over-hang which allows hybridisation of the product into the vector.

Ligation was carried out using a Takara DNA ligation kit. To 2 µl DNA 1 µl of pGEM-T Easy plasmid and 3 µl of the Takara mighty mix were added on ice. This was then incubated over night at 16 °C. The mighty mix contains a T4 DNA ligase which joins the ends of the plasmid with that of the PCR product.

2.3.3.6 Transformation

The plasmid containing the PCR product is transformed into Invitrogen Max Efficiency® DH5α™ Competent Cells to amplify the plasmid containing the DNA of interest. Blue/white selection was used to identify cells that contain the plasmid with an inserted DNA fragment. The β galactosidase enzyme normally converts its substrate X-gal to a blue precipitate in the presence of an inducer IPTG which results in the formation of blue colonies. When a DNA fragment is inserted into the MCS, the β galactosidase gene is disrupted. When this gene is disrupted the enzyme cannot convert its substrate X-gal into a blue precipitate, instead white colonies are formed.

The same transformation protocol was followed as in sub cloning, section 2.3.2.6 above, except blue/white selection could be used with the PGem-Teasy plasmid. When the LB/Amp plates were set 40 µl of 20 mg/ml IPTG and 20 µl of 20 mg/ml X-gal was spread onto each plate as they are the substrates for blue/white selection. After an hour the transformed bacteria were spread onto the plates and left agar-up at 37 °C over night. The following morning blue and white colonies of *E. coli* could be seen growing on the plates. They were left in the fridge until ready for picking for the minipreps.

2.3.3.7 Minipreps

Minipreps are carried out to isolate the amplified plasmid from the bacterial culture. Usually only 6 minipreps were needed as TA cloning usually has a high efficiency and blue/ white selection was used. 6 white colonies were picked and

the same protocol was followed as in section 2.3.2.7 to give purified plasmid DNA containing the insert of the gene of interest.

2.3.3.8 Restriction Analysis for Insert

To see if the miniprep clones contained the gene of interest 3 µl of the purified DNA was analysed by restriction digest with EcoR1 and the DNA was digested over night at 37 °C. There are Eco R1 sites on either side of the MCS of P-Gem, so the product obtained should be the same size as the product produced from the primers for the gene of interest.

2.3.3.9 Restriction Analysis for Orientation

Restriction digest was then carried out with the clones that had the correct sized insert to determine the orientation of the PCR product. As TA cloning was used the PCR fragment could be inserted in the forward or reverse orientation. The genes of interest were analysed on Vector NTI to determine what restriction enzymes can be used to produce two fragments of different size to determine what orientation the genes are in. The restriction site should be present in the PCR fragment and in the MCS to get appropriate sized products.

2.3.3.10 Glycerol Stocks

One clone showing the correct restriction analysis for each gene was used to make a glycerol stock. 850 µl of the bacterial suspension saved from the miniprep was added to 150 µl of pure glycerol in a cryovial. The stocks were flask frozen by immersion in liquid nitrogen for a few seconds, and then stored at -80°C until required.

2.3.3.11 Maxi Preparation of DNA

A maxi prep was carried out to get enough DNA for sequencing and for probe template preparation. The same procedure was followed as described in 2.3.2.9. The DNA was pellet was dissolved in T.1E or DEPC water, concentration determined on the Nanodrop and sent for sequencing.

2.3.3.12 Sequencing

An aliquot of maxiprep DNA was sent to the Waikato University DNA sequencing facility for sequencing. This is to ensure the insert is in fact the correct one and that the ligation sites are correct. Primers were supplied by the sequencing facility and used either SP6 (reverse orientation) or T7 (forward orientation) polymerases. The sequence of the insert can then be compared to that of the original by using vector NTi software. Any mismatches can be checked if they are real by looking at the chromatogram to see if an incorrect call has been made by the software.

2.3.3.13 Probe Template Preparation

To prepare a RNA probe the template must be linearised first by restriction enzymes. Restriction enzymes to use were determined by finding an enzyme cuts uniquely on the 5' end and that creates a 5' overhang. If the insert was in the forward orientation in the plasmid then the SP6 RNA polymerase was used to create an antisense RNA copy of the DNA. And if in the opposite orientation the T7 RNA polymerase was used.

10 µg of the gene of interest DNA was digested at 37 °C for 2 hrs. After 2 hr 1.25 µl of 2 mg/ml Proteinase K was added and incubated at 37 °C for a further 30 min. RNase free solutions were used from here on. After 30 min, 50 µl of TE was added and a phenol chloroform extraction was performed with through vortexing and centrifugation for 2 min at 13 K. The top aqueous layer was transferred into a new tube. Chloroform extraction was performed by adding 100 µl of pure chloroform to the supernatant tube followed by vortexing and centrifuged again. The top aqueous layer was transferred to a new tube and 10 µl of 3 M sodium acetate (pH 5) and 250 µl of 100 % ethanol was added. The solution was mixed and left at -20 °C for at least 3 hr. After this time the samples were centrifuged at 4 °C for 15 min and the pellet was washed with 70 % ethanol, centrifuged again, the ethanol was aspirated and the pellet was air dried. The pellet was dissolved in 15 µl RNase free 10 mM tris, 0.1 mM EDTA. The concentration was estimated by running a 1:10 dilution of the DNA on a 1 % gel against 2 and 5 µl of a lambda standard.

2.3.3.14 Preparation of Riboprobes

Dig probes were prepared from the following mixture:

10 x transcription buffer	2 µl
10 x Dig labelling mix (Boehringer)	2 µl
RNase Out (invitrogen)	1 µl
RNA polymerase (T7 or SP6) (Boehringer)	2 µl
Linearised DNA template	1 µg
DEPC to 20 µl	

The reaction mix was left a 37 °C for 2-3 hrs to allow the RNA polymerase produce an RNA copy of the DNA. After 2-3 hrs the RNA was run on a 1.4 % gel with a Lambda DNA ladder. A thin sharp slow migrating DNA band was expected to be seen along with two intense RNA bands that were smaller in molecular weight. If bands were what was expected they were run through a Roche quick spin column for RNA. This column works by size exclusion, where the larger molecules will be eluted at an earlier time than the smaller molecules as they get absorbed into the matrix. This allows the separation of nucleic acids from other molecules like the labelling mix. The probe was then ethanol precipitated by adding 2 µl of 4 M lithium chloride and 3 volumes of ethanol and incubated overnight at -20 °C. After this time the RNA was centrifuged at 13,000 rpm for 15 min, washed with 75 % ethanol and air dried. The pellet was resuspended in 20 µl of DEPC water. The RNA concentration of the probe was estimated by using the nanodrop and then run on a 1.4 % agarose gel.

2.3.4 Whole mount *in situ* hybridisation

2.3.4.1 Embryo Retrieval and Dissections

Wild type or +/- *Elf5* female mice were mated and checked for plugs daily (a sign of mating). The mice were then left for the embryos to develop for the required time period (6.5 or 7.5 days). Upon this time the pregnant female mice were culled by cervical dislocation or gassed with CO_2 and the uterine horns were removed and put into PBT (phosphate buffered saline with Tween). My animal

ethics application was approved on the 18/07/08 by the University of Waikato animal ethics committee, protocol number 731. The multiple decidua that contained the embryos were carefully removed from the uterine lining. The embryos were found inside the top half of the pear shaped decidua. Up to 12 embryos could be extracted from one pregnant female. The embryos were then fixed in 4 % paraformaldehyde in PBT for 2 hr at room temperature for E7.5 or one hour for E6.5. After this time the embryos were dehydrated in methanol by washing with PBT two times and then 20 %, 40 %, 60 %, 80 % and 100 % methanol. The embryos were then washed 3 x in methanol and stored at -20 °C until further use.

2.3.4.2 Hybridisation of embryo's

The method was received by personal communication from Ye at al., (1995).

2.3.4.3 Pre-treatment and Hybridisation

The embryos were rehydrated through 75 %, 50 % and 25 % methanol in PBT and then washed 2 x in PBT. The embryos were then treated with 10 µg/ml proteinase K in PBT for 5 min. Proteinase K was used to permeabilise the embryos to allow the RNA probe to enter the tissue easily to enhance the signal. Permeabilisation was not allowed to continue longer than 5 min as it can damage the embryos. They were then rinsed carefully with PBT to remove the proteinase and post fixed for 20 min in 4 % PFA containing 0.1 % glutaraldehyde in PBT. The embryos were then rinsed and washed once in PBT. They were then rinsed once in 1:1 PBT and hybridisation mix when embryos had settled (and therefore equilibrated), then once with 1 ml hybridisation mix and allowed to settle again. The hybridisation mix was then replaced with fresh mix and the embryos were incubated horizontally for 1 hr at 65 °C. The embryos were then divided into 6 tubes (for 6 different probes) and after one hour 1 ml pre warmed hybridisation mix with 1 µg/ml (~2 µl) of the digoxygenin(DIG)-labelled RNA probe was added and immediately placed at 65 °C. It was very important at this step to keep everything at 65 °C to prevent background forming. The embryos were then incubated horizontally at 65 °C over night, rocking gently.

2.3.4.4 Post Hybridisation Washes

After the overnight incubation the embryos were rinsed twice with 65 °C pre-warmed hybridisation mix. Then washed twice at 65 °C for 30 min with 1.5 ml hybridisation mix, again rocking gently. Then another wash was carried out horizontally for 10 min at 65 °C with 1.5 ml of pre-warmed 1:1 hybridisation: MABT mix. The following rinses were then carried out: 2 x with 1.5 ml MABT, 2 x with 1.5 ml $MABT_{500}$ at room temperature. The Embryos were incubated in MABT500 containing 100 µg/ml RNase A at room temperature for 30-60 min. The embryos were then rinsed 2x with $MABT_{500}$ and 2x with MABT. The embryos were then incubated for one hour in MABT containing 2 % Boehringer blocking reagent at room temperature. This was followed by an incubation at room temperature for 1 hour in MABT containing 2 % Boehringer blocking reagent and 20 % heat treated lamb serum. After an hour the embryos were incubated over night at 4 °C in fresh MABT containing 2 % Boehringer blocking reagent and 20 % heat treated lamb serum plus a 1:2000 dilution of Alkaline phosphatase-anti-DIG antibody. Alkaline phosphatase converts its substrate NBT and BCIP to a blue coloured product.

2.3.4.5 Post Antibody Washes and Histochemistry

After the overnight incubation the embryos were rinsed three times with 1.5 ml MABT and transferred to glass scintillation vials. They were then washed 3x for an hour with 10 ml MABT by rolling. And then washed two times, for 10 min with 10 ml NTMT. For colour development of the probe the embryos were incubated at room temperature with 1.5 ml NTMT + 4.5 µl/ml NBT (75 mg/ml in 70 % Dimethyl Formamide) + 3.5 µl/ml BCIP (X-phosphate; 50 mg/ml in 70 % DMF). The embryos were left in the dark for the colour to develop. Colour development took up to three days. When the colour had developed to the desired extent the embryos were rinsed 2x in PBT and transferred to an 80 % glycerol solution and kept at 4 °C in the dark.

Photographs of the embryos were taken using the Leica DMI6000B microscope and camera.

2.4 Statistics

Whole mount in situ hybridisation falls outside statistics as it is a picture of fixed embryos, it cannot be quantified. Gene expression by quantitative real time PCR (qRT-PCR) was normalised against three house-keeping genes (GAPDH, β-tubulin or β-actin). The geometric mean of the three housekeeping (HK) genes was taken to average out the effect of the house keeper ($\sqrt[n]{}$ (multiplication of nHK)). All samples had biological repeats in either duplicates or triplicates and triple qRT-PCR measurements were taken to calculate the standard error of the mean (SEM). Significance was calculated by Students t-tests.

3 Chapter Three: Elf5 target genes in mouse Trophoblast Stem Cells

3.1 Introduction

Elf5 is a transcription factor that is essential for the maintenance of the extra-embryonic ectoderm and the trophoblast stem cells that reside within. The role of Elf5 can be delineated by understanding the genes that it controls during early development. A common method used to delineate targets of genes is to knock the gene down *in vitro* by RNA interference (RNAi). Once the gene is knocked down potential down-stream targets can be identified by virtue of their change in expression.

Such a global analysis of gene expression was carried out for Elf5 by Deane (2007) using an microarray (affymetrix). The expression of 22 genes were changed in common by two independent short interfering RNA molecules (siRNA) targeting *Elf5* mRNA. I have analysed these candidate target genes further, by repeating the knockdown and analysing expression at two time points (24 and 48 hr post siRNA treatment) using quantitative real time RT-PCR to measure expression levels.

3.2 Background to technology used

3.2.1 RNA Interference

RNA interference is a highly specific and powerful gene silencing event that is carried out by double stranded RNA. RNA interference was discovered when researchers Andrew Fire and Craig Mello who were studying larval development in the nematode worm *Caenorhabditis elegans,* discovered a gene *lin-4* encoding short RNA transcripts, inhibited the translation of another gene, *lin-14* (Lee et al., 1993). RNA interference is wide spread and is seen in plants, fungi and animals. Small RNAs like micro RNAs (miRNA) and small interfering RNAs (siRNAs) have important and wide spread functions including development, disease, apoptosis and cancer (Rana, 2007). Small interfering RNAs (siRNA) and miRNAs are 19-25 base pairs long and use the same machinery to elicit a gene silencing response.

SiRNAs have not been found to be produced endogenously in cells but they can be introduced into cells and use miRNA machinery. Both RNAs induce post transcriptional gene silencing of their targets by complementary binding to target mRNA sequences and causing either cleavage or translational inhibition.

Small interfering RNAs can be introduced exogenously to cells to provoke RNAi. SiRNAs induce gene silencing by causing specific degradation of the target sequence upon recognition. The target sequence is only degraded if prefect binding is achieved. Micro RNAs do not cause degradation of their targets as perfect complementarity is not achieved, instead translation is suppressed (Rana, 2007). Small interfering RNA is double stranded, one strand is the sense strand and the other is the antisense or guide strand. The guide strand is incorporated into the RNAi machinery, the RNA induced silencing complex (RISC) to silence the target mRNA. The sense strand of the siRNA is degraded. The RISC complex containing the guide strand binds to the target mRNA and prevents the expression of that gene by cleavage of the target mRNA (Rana, 2007).

The function of genes can be elucidated by targeted silencing *in vitro* or *in vivo*. This can be achieved by the addition of exogenous siRNAs to knock down the gene of interest. After the gene of interest is knocked down the down-stream effects can be analysed. There are many problems associated with RNAi, including achieving a high transfection efficiency, specificity and activity (Hough et al., 2006). The 'seed region' which is base 2-8 from the 5' end of the guide strand of the RNA is very important for target site recognition (Rana, 2007). There are two major factors that must be taken into account with regard to siRNA specificity; off target effects due to mRNAs with similar sequence as the target mRNA and immune stimulation (Bumcrot et al., 2006). RNAi silencing of target genes can be very specific, but off target effects can occur due to sequence similarity of mRNAs particularly at the 3' end that binds to the seed region of the siRNA (de Fougerolles et al., 2007). Chemical modification of the riboses of the guide strand can reduce off target effects. In particular 2'-O-methyl modification at nucleotide 2 suppresses most off target effects without affecting the suppression of the desired target (Bumcrot et al., 2006; de

Fougerolles et al., 2007; Jackson et al., 2006). Off target effects can also occur due to the sense strand being incorporated into the RISC machinery instead of the anti sense strand. It has been revealed that designing siRNAs with weaker binding at the 5' end of the desired guide strand increases specificity as the RISC machinery will preferentially incorporate that strand. This can be accomplished by making a nucleotide substitution at the 5' end (Bumcrot et al., 2006; de Fougerolles et al., 2007).

Immune stimulation can be caused by interaction with toll like receptors resulting in the production of interferon. Sequences that have similarity to toll like receptors should be avoided, 2'-O-methyl modifications within the siRNA also prevent most immune responses (de Fougerolles et al., 2007). Most companies that offer siRNAs have algorithms that can design siRNA duplexes with these specifications.

An optimal transfection efficiency depends on many things including cell density, RNA concentration, lipofectamine concentration and media components (Dalby et al., 2004). As nucleic acids and cell membranes are negatively charged something is needed to mediate transfection to overcome the electrostatic repulsion. Lipofectamine is a positively charged liposome that can complex with RNA to facilitate up take into cells (Dalby et al., 2004). The lipofectamine complexes with the RNA and upon addition to cells, fuses with the cell membrane to release the RNA into the cell. Serum and antibiotics in the media can also reduce the transfection efficiency (Dalby et al., 2004).

3.2.2 Quantitative Real time PCR

Quantitative real time reverse transcribed polymerase chain reaction (qRT-PCR) is used to detect and quantify messenger RNA (mRNA) in samples. Real time PCR permits the detection of the PCR product during the exponential phase of amplification allowing the simultaneous detection and amplification in one (Giulietti et al., 2001). The fluorescent signal increases as the DNA is being

amplified in each PCR cycle. qRT-PCR can be used to compare the expression of mRNA in different tissues, different sample treatments and can detect RNA from as little as one cell or embryo and is therefore a very sensitive technique (Bustin, 2000). As well as its sensitivity, no post PCR processing is needed unlike conventional reverse transcriptase PCR (Giulietti et al., 2001).

Before DNA can be amplified with a real time PCR machine, mRNA must be first isolated and converted into cDNA via reverse transcription. RNA isolation is carried out using TRizol reagent (invitrogen) and then copy DNA is made using superscript III reverse transcriptase (invitrogen). Reverse transcriptase is an enzyme purified from *E. coli* which synthesises a single strand of DNA that is complementary to the RNA. Oligo dt primers are used to amplify mRNA as mRNA contains a poly A tail at the 3' end. Oligo dt primers contain a string of T's on the end to prime to the complementary mRNA. This allows the amplification of mRNA only; this is useful as mRNA contains the sequence of a gene that will then be expressed in the form of a protein or enzyme. Once the cDNA is made specific DNA can be amplified using real time PCR.

There are numerous chemistries available for the detection of the target DNA product that is formed. The one used in our lab is SYBR green. SYBR green is a double stranded DNA binding dye that exhibits little fluorescence when in the presence of single stranded RNA or DNA, but when incorporated into double stranded DNA during the elongation step emits fluorescence. The fluorescence is measured in real time after the elongation step of every cycle to give the amount of DNA that is present (Bustin, 2000). The intensity of the fluorescence is proportional to the amount of DNA produced. The amount of starting material can be determined by the threshold cycle (C_t). The C_t value is determined by the cycle that the fluorescence of the individual sample passes a defined threshold or background fluorescence value (Nolan et al., 2006). The higher the amount of starting DNA the lower the C_t value obtained. The Ct value is the cycle number that the fluorescence level crosses the threshold (Giulietti et al., 2001). SYBR green is the most commonly used material as it can be used with any primer pair. A disadvantage of SYBR green is that SYBR green will also bind to non specific

products and primer dimers. To ensure products are specific a melt curve analysis is performed. This is achieved by plotting fluorescence against melting temperature of the amplified product. A single peak should be obtained that is characteristic of the product (Bustin, 2000). Primer dimers can be excluded as they have a lower melting temperature.

To quantify the results obtained the comparative threshold method is used. This is when formulas are used to calculate relative expression levels compared to some sort of calibrator like an untreated sample or control (Giulietti et al., 2001). The gene expression of the treated sample is compared to the untreated control to give a fold change in gene expression. Normalisation must be carried out to account for differences in starting material between samples, variation in amplification efficiencies and differences between samples (Bustin et al., 2005). This is achieved by normalisation to a house keeping gene. A house keeping gene should be expressed constitutively in a cell and should not be affected by different treatments. Common house-keeping genes include glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin and ribosomal RNA. GAPDH is an ubiquitous glycolytic enzyme present in most cell types and β-actin encodes a cytoskeleton protein (Giulietti et al., 2001). There is no single RNA that is an ideal house-keeping gene so for greater accuracy the geometric mean of three housekeeping genes is commonly used (the geomean) (Bustin, 2000).

3.3 *Elf5* Knockdown in mouse trophoblast stem cells

3.3.1 Overview

Extra cDNA samples as used for the affymetrix array were analysed for changes in gene expression of some of the Elf5 targets. For further verification the knockdown in mouse trophoblast stem cells was repeated and the target genes were analysed by real time PCR. Before a successful knockdown could be obtained, the transfection had to be optimised. A successful *Elf5* knockdown of greater than 80 % was achieved with optimisation, allowing analysis of the Elf5 targets by real time PCR. This provided further verification of those genes as Elf5 targets in mouse trophoblast stem cells.

3.3.2 Verification of microarray samples (Pilot experiments)

Two *Elf5* mRNA targeted siRNAs were shown previously (Deane, 2007) to give greater than 80% knockdown of *Elf5* in mouse trophoblast stem cells following 24 hr of transfection. The two siRNAs that were used were 337 and 733 (refer to Table 1). The name of the siRNA corresponds to the base pair position of the start of the siRNA on the *Elf5* mRNA. The sequences of the siRNAs are given below.

Table 1: Sequence of siRNA molecules used to knockdown *Elf5* in mouse trophoblast stem cells.

Start Position	Sense RNA Sequence 5'-3'	GC%
337	CCAACUGCAUCUCCUUCUGUCACUU	48
733	GGAAGAAGAAUGACAGGAUGACGUA	44

Real time PCR was carried out on some of the remaining cDNA from the affymetrix array. These samples were extra cDNA samples that were produced in the same knockdown experiment as the samples that were used for the affymetrix array. To confirm the affymetrix array a quantitative technique was also used to study the genes not already examined by Deane (2007).

The two samples that were used to knockdown *Elf5* were 337 Elf5 KD and 733 Elf5 KD. The controls were an untreated sample (no treatment) and a scrambled siRNA with no targets in mammalian cells (si negative control). The effect of differentiation/removal of growth factors was also included. When growth factors were removed *Elf5* expression decreased, see Figure 6.

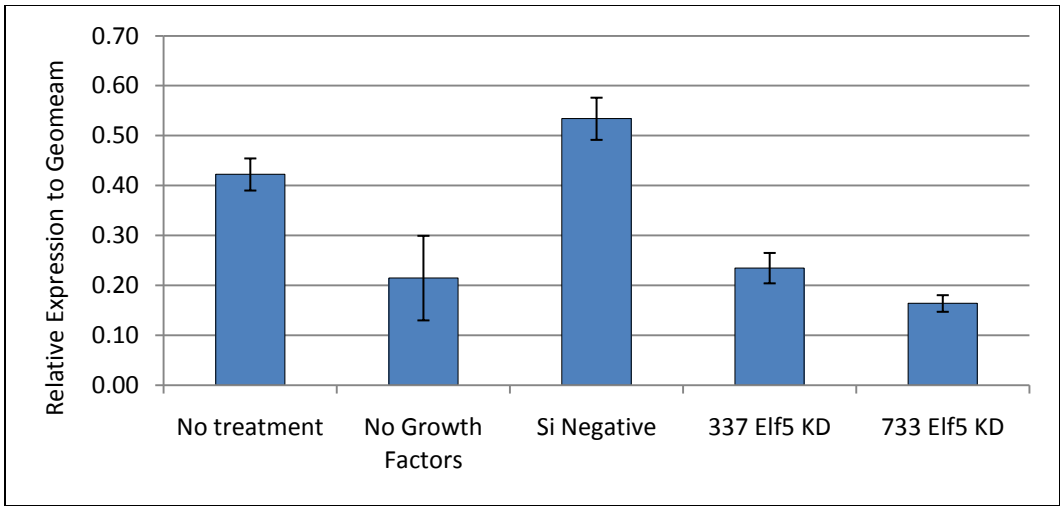


Figure 6: The level of *Elf5* Knockdown obtained by the 337 and 733 siRNAs and with no growth factors. N= 3. A scrambled oligonucleotide was included as a negative control (si negative). Expression is shown relative to the geometric mean of Gapdh, β-actin and β-tubulin.

The changes in expression of those target genes not analysed by Deane (2007) were examined by real time PCR. This was to verify the original microarray samples by the use of a different technique and for more of the identified genes. Results are shown in Table 2 below as a fold change in expression compared to the untreated control. The average of three individual biological replicates was taken. For comparison to the microarray refer to Table 3. These genes were affected only by the 733 siRNA. The results are shown as the fold change compared to the untreated control (N), see Table 2.

Table 2: Verification of the original microarray samples on genes not already examined in Deane (2007), results shown as fold change compared to the untreated control (N). n=3.

	Up or Down	No GF vs. N	337 vs. N	733 vs. N	N vs. Si-
Elf5	-	2.0	1.8	2.6	1.3 (+)
Mme	-	3.3	1.4 (+)	2.4	1.8 (+)
Ly6a	+	9.1 (-)	1.1	2.9	1.3
Elavl2	-	3.8	1.3 (+)	1.2	1.2 (+)
Zic3	-	5.4	1.3 (+)	1.4	1.4 (+)
Keratin7	+	1.2	1.1	1.9	1.1
Bex1	-	1.1	1.9	1.4	1.1
HOPX	+	3.9 (-)	1.3 (-)	2.1	1.1 (-)
1700112C13RIK	-	6.7	1.3 (+)	1.9	1.5 (+)
GM784	-	4.3	1.2	2.2	1.1 (+)

3.3.3 Knockdown of *Elf5* in TS cells

To further this investigation we wanted new samples that were independent to the affymetrix array data. Furthermore two different time points were to be investigated, 24 and 48 hr following the initial transfection. This will provide independent verification of the affymetrix array data and provide more information about the target genes and the time response to the knockdown of *Elf5*.

3.3.3.1 Replacement of Embryonic Fibroblast Conditioned Media with Activin

TGFβ and activin are key components of embryonic fibroblast conditioned media. Erlebacher et al,. (2004) demonstrated that EFCM can be replaced by TGFβ or activin (while still supplementing with Fgf4 and heparin). The TS cells could be cultured in this media without any apparent changes in their stem cell characteristics. As this is a more controlled way of maintaining TS cells (the components of each batch of conditioned media (CM) can be quite variable) we were quite interested in using activin in our media instead of CM (refer to 2.1.2.3 in methods). The production of conditioned media is also a lengthy process (refer to section 2.1.2 of methods) so the use of a commercial product was desirable. To ensure the gene expression of the TS cells didn't change in any way upon switching to activin the expression of TS cell marker genes were analysed in four

lines grown in CM and compared to four lines grown in activin. The genes were analysed by real time PCR and these were *Elf5, Eomes, Cdx2, Fgfr2* and *Bmp4*.

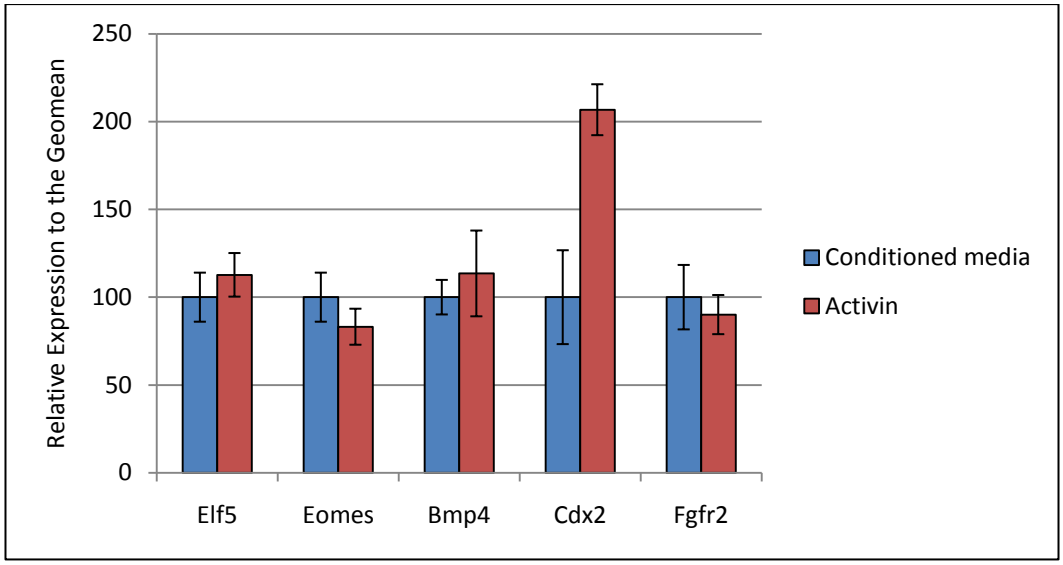


Figure 7: Gene expression profile of trophoblast stem cells grown in activin vs. conditioned media, N=4. There was no significant difference in gene expression of any gene grown in conditioned media compared to activin. Expression was normalised to the geomean and then compared to the conditioned media sample (100 %).

There was no obvious change in gene expression apart from the up-regulation of *Cdx2* in the activin treatments (see Figure 7above). Two samples in the activin treatments had high *Cdx2* levels while two other samples had similar levels to conditioned media samples. This change was not significant due to the high variation of expression in each sample. These samples were cultured at different time points and were in different RNA isolation and cDNA synthesis runs so that may have accounted for some of the variability between the samples in the same treatment. The experiments from here on were grown in activin instead of conditioned media.

3.3.3.2 Initial Knockdown of *Elf5*

To confirm the Elf5 target genes, *Elf5* was knocked down in trophoblast stem cells for 24 and 48 hr. This was followed by real time PCR to analyse gene expression of *Elf5,* and the effects of *Elf5* knockdown on the genes that were identified as target genes by the microarray.

The knockdown of *Elf5* was initially carried out in 12 well (4 cm^2) plates. For a 12 well plate 40 pmol of siRNA is used per well plus 2 µl of lipofectamine 2000 as in 2.1.3.4. The siRNA treated cells were harvested in 200 µl Trizol after 24 or 48 hr. The greatest knockdown of *Elf5* achieved using this protocol was only about 50-60 %. As we wanted knockdown of *Elf5* of at least 70-80 %, optimisation was clearly needed. Two different siRNAs were used to knockdown *Elf5*- 337 and 733. The controls were the si negative control which was a scrambled siRNA with no mammalian targets, and an untreated control. A sample was also added with the removal of growth factors to see what effect differentiation has on gene expression.

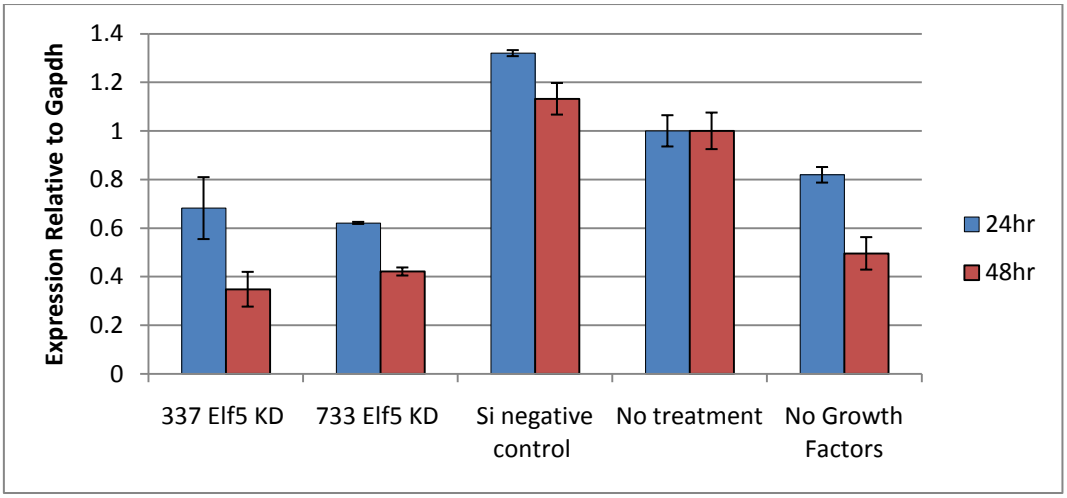


Figure 8: The best knockdown of *Elf5* achieved before optimisation in mouse trophoblast stem cells. At 24 hr only 40 % knockdown of *Elf5* was achieved, after 48 hr 60-65 % knockdown was obtained by both siRNAs compared to the no treatment control. Expression was normalised to the house keeping gene Gapdh.

3.3.3.3 Transfection efficiency and optimisation of seeding density with RNAiMAX

The optimisation of seeding density was considered first, this is because seeding density can severely affect the transfection efficiency. According to the invitrogen lipofectamine 2000 transfection protocol (2002) a seeding density of 30 to 50 % confluency was optimal for a transient transfection. An optimal seeding density in 12 well plates was difficult to obtain due to the small surface area. The cells tended to clump in the middle making it hard to achieve 30- 50 % confluency at the time of transfection. For a 48 hr long transfection the cells

were passaged at a lower density than those that were left for 24 hrs, as the cells were far to over grown if seeded at the same density as a 24 hr long transfection. In 12 well plates only an *Elf5* knockdown of 60 % or less was ever obtained, see Figure 8. During this time a seeding density of $2x10^4$ cells/cm^2 for a 24 hr transfection and $1.5x10^4$ cells/ cm^2 for a 48 hr transfection seemed optimal.

The transfection efficiency of the RNA was investigated by transfecting the TS cells with a Block-iT Alexa Fluor Red Fluorescent oligonucleotide (Invitrogen) which is essentially an oligonucleotide (oligo) coupled to a fluorescent compound. At the same time a novel type of lipofectamine, RNAiMax (invitrogen) was also tested. Both forward and reverse transfections were carried out. For a reverse transfection the cells were plated at the time of transfection (not the day before as in a forward transfection). Two different seeding densities were also investigated.

For the reverse transfection 12 pmol of the fluorescent oligo was combined with 200 µl of optiMEM. 2 µl of RNAiMAX was added to the optiMEM/oligo mixture and was incubated for 20 min. A confluency of 30-50 % of cells was desired after plating so 40,000-100,000 cells per well were needed. 1 ml of cells was combined with 200 µl of the Lipofectamine/RNAi mixture and the cells were plated down. For a forward transfection 12 pmol of RNAi was added to 100 µl optiMEM, and 2 µl of lipofectamine or RNAiMAX was added to 100 µl optiMEM. After 5 min the two were combined and incubated for 20 min (refer to 2.1.3.2 of materials and methods). Then the mixture was added drop wise to the cells. The cells were left to incubate with the siRNA for 4 hr and then the media was replaced. After 24 hr the cells were washed with PBS and then fixed with paraformaldehyde. The transfection efficiency was then observed with a LEITZ DM IRB microscope (Leica). The reverse transfection did not work, as no fluorescence was observed. No fluorescence was observed using the RNAiMAX lipofectamine either. Due to the failure of the reverse transfection and the new lipofectamine, the results were only comparing two different seeding densities of a forward transfection. A lower seeding density appeared to result in a higher transfection efficiency, see Figure 9.

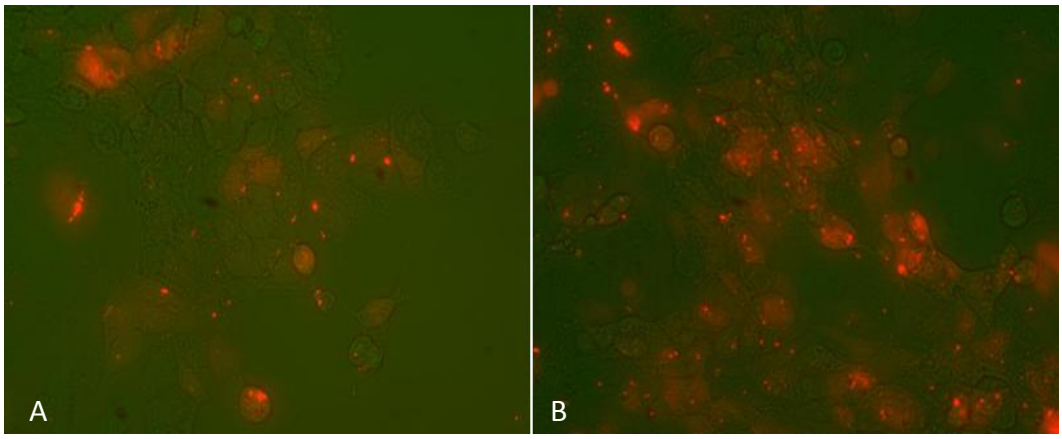


Figure 9: TS cells transfected to optimise the seeding density with an Alexafluor oligonucleotide, Transfection at a higher seeding density A), and transfection at a lower seeding density B). A 10x magnification was used. The fluorescence was artificially coloured red and the cells green. Photographs were taken using a LEITZ DM IRB microscope (Leica).

3.3.3.4 Optimisation of siRNA and Lipofectamine Concentration

The forward transfection above was repeated while optimising for lipofectamine 2000 concentration, seeding density and siRNA concentration. The results were quite subjective as they were analysed by light microscopy, possibly a better transfection efficiency was achieved using twice the amount of lipofectamine, see Figure 10. The results depended on what colony was photographed and exposure times. Photographs were taken using a LEITZ DM IRB microscope (Leica).

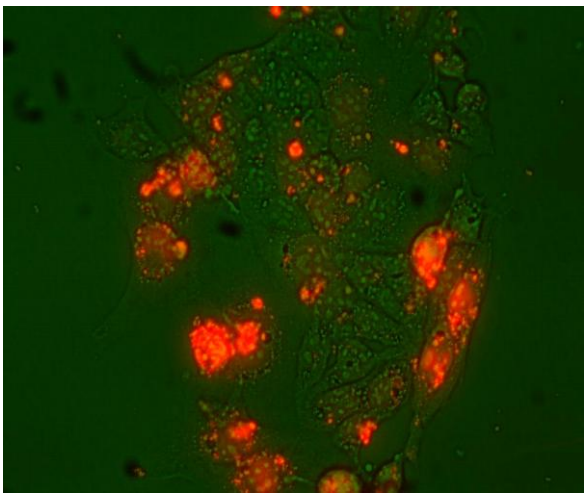


Figure 10: Transfection with 12 pmol of an alexa- fluor fluorescent oligonucleotide with a double lipofectamine concentration to determine the transfection efficiency. Photographs were taken at a 10x magnification. The fluorescence was artificially coloured red and the cells green. Photographs were taken using a LEITZ DM IRB microscope (Leica).

3.3.3.5 Flow Cytometry to Determine Transfection Efficiency

As the microscopy results were quite subjective, flow cytometry (FACS) was used to determine the transfection efficiency of the mTS cells with the fluorescent oligo. For FACS analysis $2x10^5$ to $1x10^7$ cells were required. Due to this the transfection was shifted to a 6 well dish to obtain an adequate number of cells to analyse. Firstly unlabelled cells were used to give an idea of the cell population that was being studied. One 50 % confluent well of a 6 well plate was washed 2 x with PBS, trypsinised, and re-suspended in PBS containing 1 % FCS to prevent cell clumping. The cells were passed through a 35 µm filter (BD) to remove any cell clumps as only one cell at a time can pass through the sample injection port (SIP). Forward scatter and side scatter settings were optimised for the cells on the Becton Dickinson FACScan™.

Once the machine was optimised for the cell type, the cells were transfected as per usual (section 2.1.3.4) but the reaction was scaled up to account for larger volumes. Only one concentration was used to optimise the machine and then the transfection conditions were to be optimised at a later time. 50 nM siRNA plus 10 µl lipofectamine 2000 was used. Cells were washed and trypsinised after 24 hr as above. The fluorescence was captured on FL2 channel. Of the gated cells 91.83 % of the population (cells of interest) were fluorescing, see Figure 11. This meant that the transfection efficiency was already very high and there was no need to optimise the transfection conditions. Region 1 (M1)/purple histogram contained the unlabelled cells, there was no fluorescence. When the cells were transfected with the fluorescent oligo the majority shifted to region 2 (M2), which demonstrated that there was a high transfection efficiency. 91.83 % of cells were in the M2 region which means these cells had been transfected with the fluorescent oligo. The peak was quite broad which implicates that different cells were transfected with varying amounts of the fluorescent oligo. When the TS cells were transfected under these conditions a knockdown of greater than 80 % was obtained (see Figure 12). This was presumably due to doubling the lipofectamine 2000 concentration and having the cells evenly spread out in the dish which makes them more accessible to the siRNA.

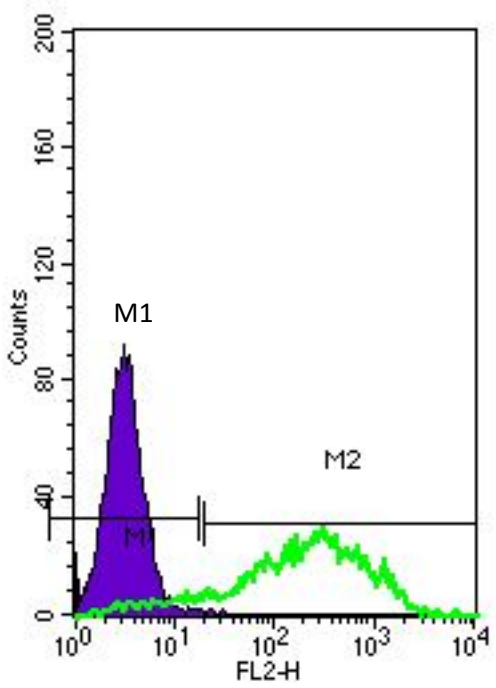


Figure 11: Transfection efficiency determined by flow cytometry. Region M1 contains the untransfected cells; there was no fluorescence as the cells were in the 10^0-10^1 region on the histogram (purple shading). When the cells were transfected with the Alexa-fluor oligonucleotide 91 % of the cells shifted to region 2 (green line). This indicated a high transfection efficiency was achieved.

3.3.3.6 Transfection of mTS cells with Elf5 siRNA

As a 92 % transfection efficiency was obtained in 6 well plates all transfections were carried out in 6 well plates hereafter. The TS cells were grown in activin for these experiments. The reactions were scaled up accordingly. 50 nM of siRNA was combined with 250 µl optiMEM and 10 µl of lipofectamine 2000 with 250 µl optiMEM. The mixtures were combined after 5 min and then incubated for 20 min at room temperature. During this time the media in the plates were replaced with 1.5 ml 5 % FCS media (no antibiotics). After 20 min 500 µl of the siRNA/lipofectamine mixture was added drop wise to the cells.

The Elf5 oligos 337 and 733 were added in triplicates and the siRNA negative oligo and no treatment cells were prepared as duplicates as they act as duplicates for each other. The removal of growth factors was included to determine the effect of differentiation on *Elf5* and its target genes. A 24 hr or 48

hr after the beginning of the transfection 500 µl Trizol reagent was added to each well. The RNA was extracted (see 2.3.1.1 and cDNA synthesised (see 2.3.1.3).

Real time PCR was carried out on the 24 hr and 48 hr samples separately according to the protocol outlined in 2.3.1.5. Gene expression was normalised to the geometric mean of three house-keeping genes, *GAPDH, β-actin and β-tubulin*. A greater than 80 % knockdown of *Elf5* was achieved 24 and 48 hr following the siRNA transfection. Expression of *Elf5* is shown relative to one of the no treatment samples, see Figure 12 below.

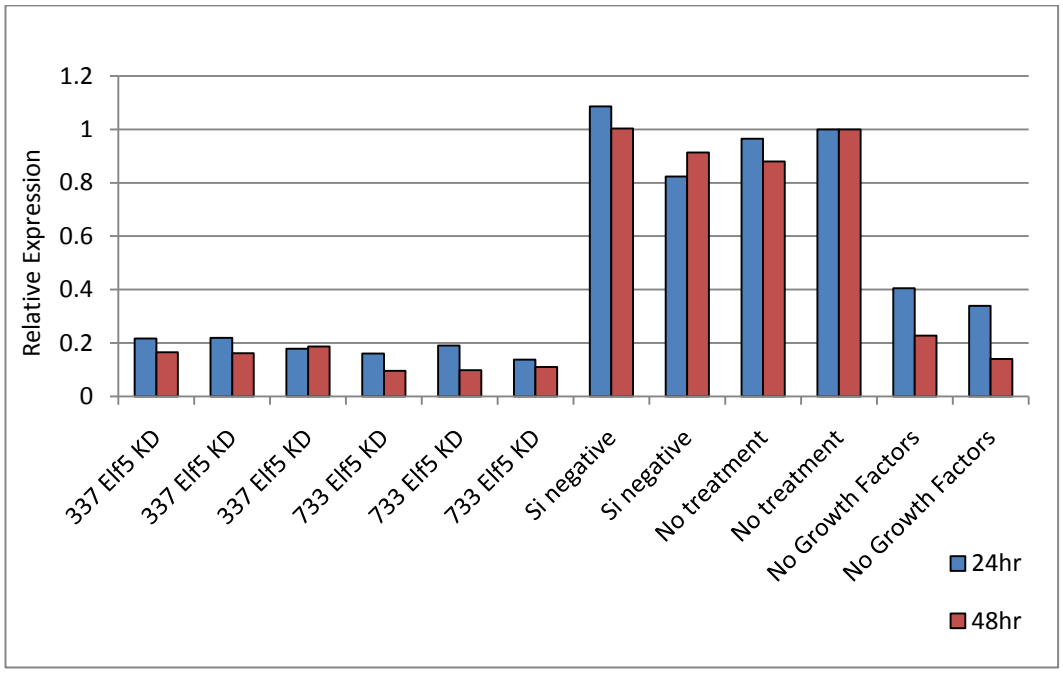


Figure 12: A >80 % knockdown of *Elf5* was achieved after 24 and 48 hr transfection, if averaged both 337 and 733 are significantly different to the si negative control p<0.005 for 24 hr and p<0.0002 for 48 hr. The expression was normalised to the geometric mean of the three housekeeping genes, *Gapdh, β-actin* and *β-tubulin*. Expression is then shown relative to one of the no treatment samples.

3.3.4 Genes of Interest Identified by the Affymetrix Array

The genes of interest identified by the affymetrix array were summarised in a table showing the fold change of every probe set compared to the control upon the knockdown of *Elf5*. Table 3, shows several of the genes of interest identified by the affymetrix array. The text in red shows the genes that are affected by both siRNAs and growth factor removal, the text in blue is affected by both siRNAs but

not growth factor removal and the purple text is affected by only 733 and by growth factor removal.

Table 3: List of genes identified by the affymetrix array as possible Elf5 target genes. Data provided by Deane (2007). The results are shown as a fold change compared to the no treatment sample (N). Red= affected by both siRNAs and growth factor removal, blue = both siRNAs but not growth factors and purple is affected by only 733 and growth factor removal.

Genes of Interest		Up or Down	No GF vs. N	337 vs. N	733 vs. N	N vs. Si-
Cyr61	IGFBP,cysteine rich protein 61	-	2.0	1.3	2.0	1.2
Sox2	high mobility group box protein SOX2	-	8.6	1.4	1.9	1.0
WDR40B	WD repeat domain 40B	-	3.3	1.9	2.1	1.3
Synpo2l	Synaptopodin 2-like protein	-	4.2	2.1	2.4	1.1
Mme	Neprilysin (EC 3.4.24.11) (Neutral endopeptidase) (NEP) (Enkephalinase) (Neutral endopeptidase 24.11) (Atriopeptidase) (CD10 antigen).	-	2.4	1.3	2.3	1.1
CALCB (incl EG:797)	Alpha Cgrp,CA,Cal1,CAL6,Calc,Calc1,CALC2,Calca,CALCB,Calcitonin,CGRP,Cgrp alpha,CGRP-1,CGRP-II,CGRP2,Ct,Ctn,FLJ30166,MGC130530,RATCAL6	-	2.0	1.5	1.7	1.0
Ly6a	lymphocyte antigen 6 complex, (Sca1)	+	5.3	1.2	2.2	1.1
Sct	secretin	+	2.2	1.5	2.2	1.2
Elavl2	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	-	2.1	1.0	1.8	1.1
Spi8	serine protease inhibitor 8	-	2.1	1.2	1.7	1.1
Hmga2	high mobility group AT-hook 2	-	1.6	1.1	2.0	1.1
Zic3	Zic family member 3 heterotaxy 1 (odd-paired homolog, Drosophila)zinc finger protein of the cerebellum 3	-	7.5	1.0	2.1	1.1
KRT7	keratin 7	+	1.8	1.2	2.1	1.1
Rex3	Bex1	-	1.3	1.4	2.1	1.1
HOPX	HOP homeobox	+	2.2	1.0	1.6	1.1
1700112C13RIK	RIKEN cDNA 1700112C13 gene	-	3.0	1.2	2.1	1.1
GM784	gene model 784, (NCBI)	-	3.3	1.6	2.5	1.2

The genes in the table above were identified by the affymetrix array as possible Elf5 target genes. Real time PCR was carried out only on these genes upon *Elf5* knockdown as these were the main genes of interest. Other possible targets were identified but they were eliminated due to low expression levels, or a high si negative effect.

Primers were designed according to the outline on 2.3.1.4, the primer sequences used are given in Table 4 and Table 5 below. The primers were tested via reverse transcriptase PCR and the product was run on an agarose gel to ensure a product of the correct size is obtained (refer to sections 2.3.1.8 and 2.3.1.9 of methods).

All primers gave one PCR product of the correct size so they were able to be used in real time PCR.

Table 4: Sequences of the primers used to amplify the housekeeping genes

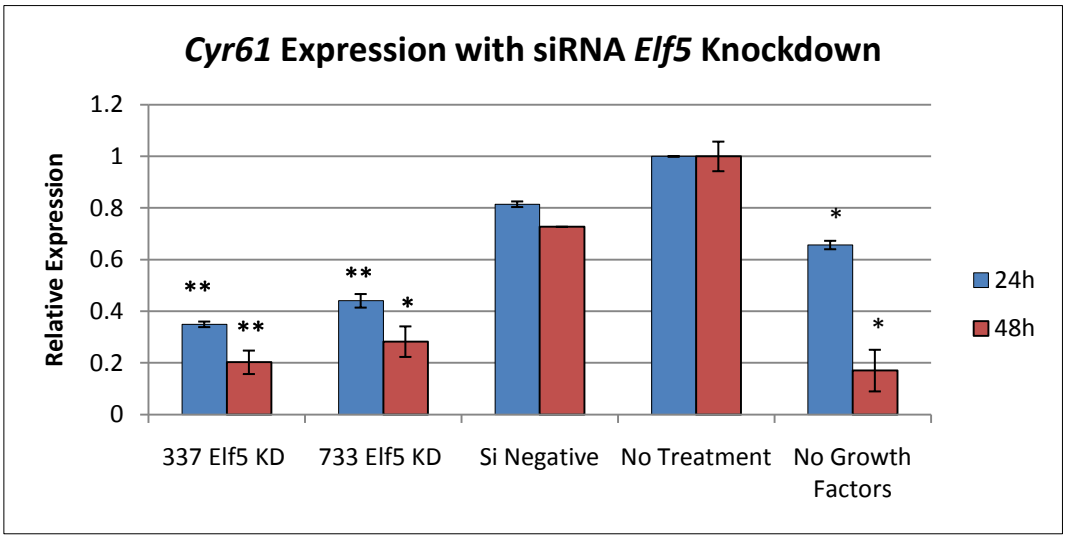
House Keeping genes	Forward, reverse 5'-3'	Amplicon Size	No. of introns spanned	GC rich
Gapdh	TGCACCACCAACTGCTTAG, GATGCAGGGATGATGTTC	175	1	No
βactin	CAGAAGGACTCCTATGTGGG, TTGGCCTTAGGGTTCAGGG	200	1	No
βtubulin	TGTAGAACCCAGCCCAATTC, CCTGAGGCTGAGAGATGAGG	200	1	No

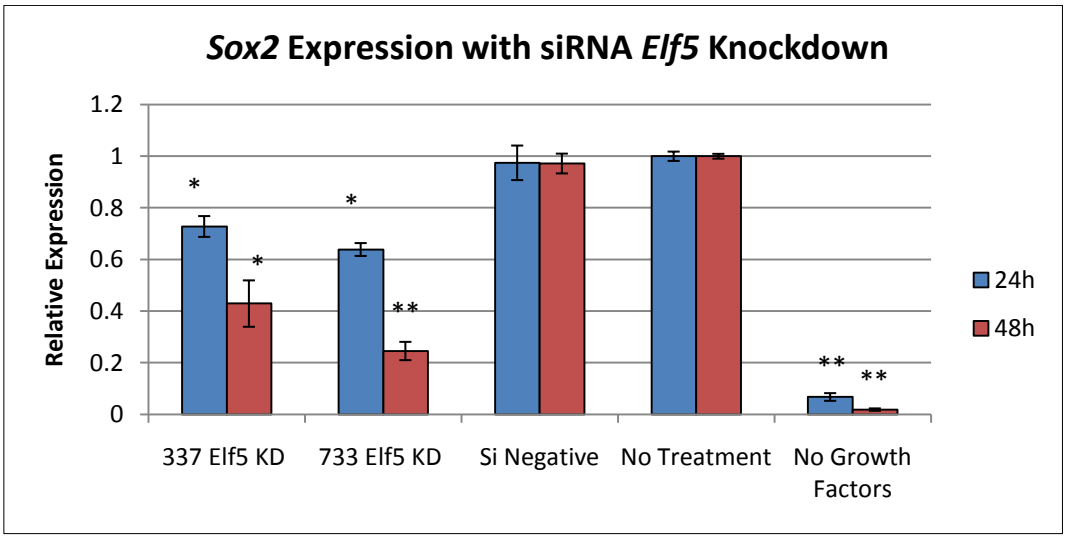
Table 5: Sequences of primers used to amplify the genes of interest by real time PCR. The GC rich genes have to have 1x GC rich solution included in the PCR mix.

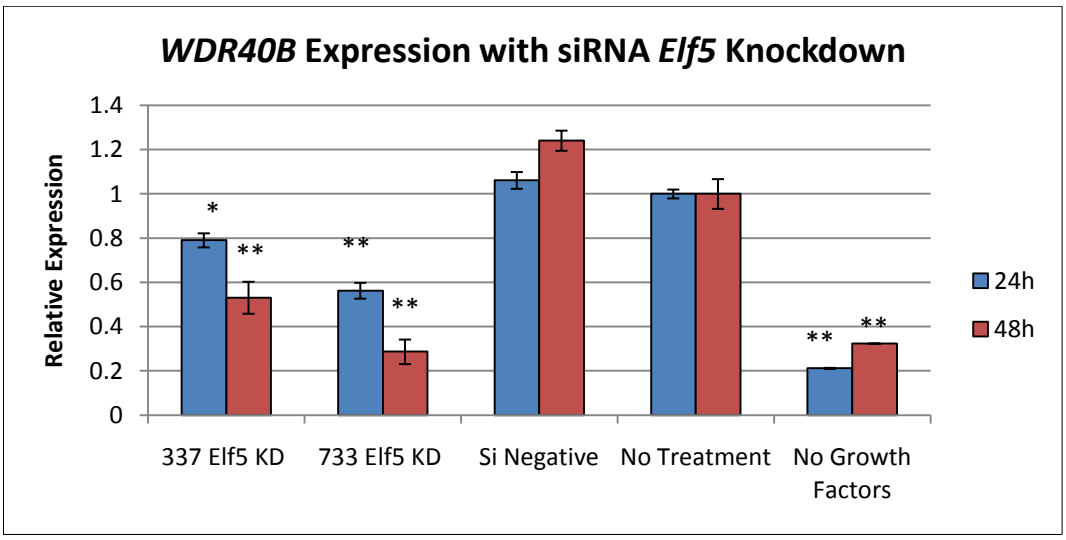
Gene	Forward, Reverse 5'-3'	Amplicon Size	No. of introns spanned	GC rich
Cyr61	AAAGAGACCCGGATCTGTGAA, TCCATCTTCGCATCGGAA	237	1	No
Sox2	GAGCTAGACTCCGGGCGATGAA, CACGAAAACGGTCTTGCCAGTA	279	1	No
Calca	AGCAGATCAGGAGGTGTGGTGAA, AGCCAGAACCATGCTGTCATGT	335	1	No
Wdr40B	TGAGCCTATCCAGCTCACTG, CACGGCATCCCATTATCTCT	253	2	No
Hmga2	AGCCGAGGTGCATTATCTTG, GGTTTGGTTTGGTTTTGGTG	201	1	No
Spi8	TATCTGGCACCACAAAACCA, AGGCAGAGTCCCCTGTATCA	249	0	No
Secretin	CGATGCTACTGCTGTTGCTG, CCAGGCTGTTCTCTGGGATA	201	1	Yes
Bex1	GAAGAGCGGAGCAGGTCTGAGAA, ACCCTCTGCATCAGGTCCCA	314	2	No
Elavl2	AAGCGGATTGAGGCAGAA, GTTGTACACAAATATGCACC	325	2	No
Mme	ACGCAGCAGTCGGCAAATAA, GCAAAGTCCCAATGATCCTGA	349	4	No
Gm784	CCCTTGCTTGCGCTATGAA, CCAGTGATAGGAGAGCCATGA	246	1	No
Ly6a	CCA TTT GAG ACT TCT TGC CCA TC, GGTCCAGGTGCTGCCTCCATTG	243	1	No
Zic3	CGAGTGCACACTGGCGAGAA, CATGTGTTTGCGCAGGGA	264	2	No
Kert7	AGACGGAATGGGACCTGTGAA, CAGGGACATGAGATGGACAAA	244	1	No
1700112c13	TTGGCTGTCAGGATTGTGAA, AGTCAAAGTTCCAGCTCACCA	211	1	No
Hopx	GGTGGAGATCCTGGAGTACAA, GAGATGGAGTTCCAAGAGCAA	223	1	Yes
Synpo2L	TCACTACCACCTACACCCAGAA, CCGCCGCTTCTTAAACAT	170	1	No

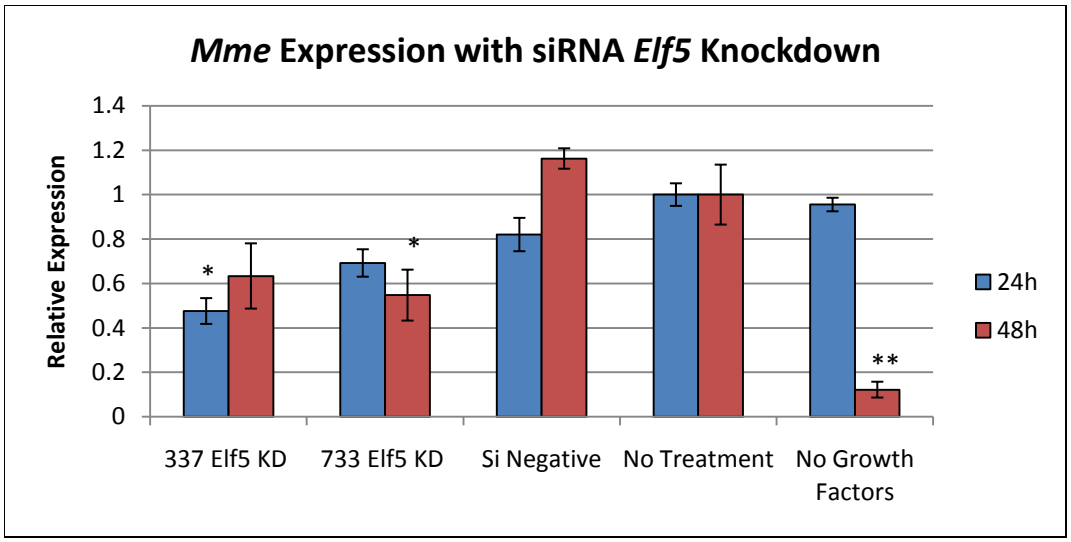
3.3.5 Analysis of Target Genes

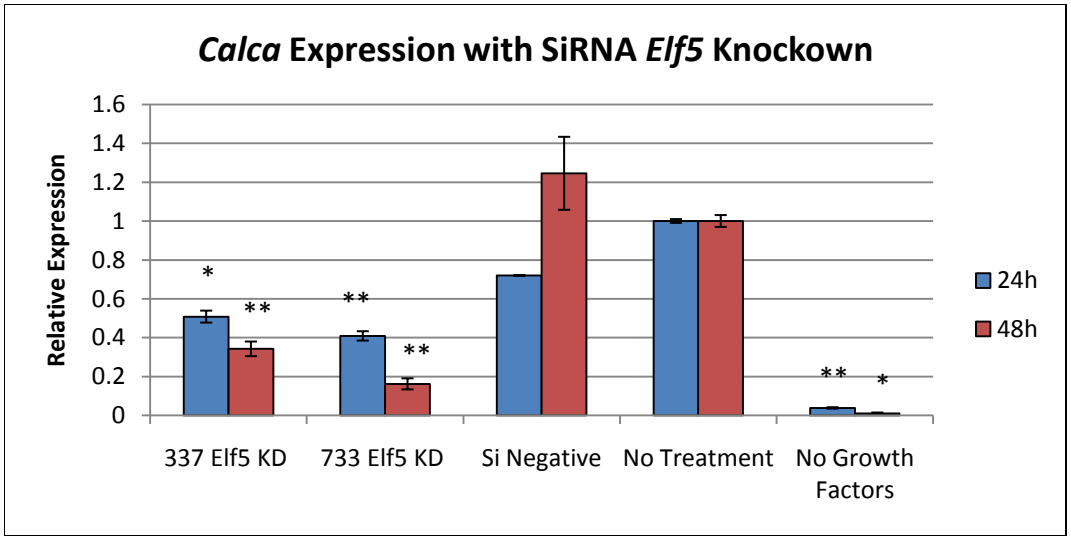
As a good knockdown of *Elf5* was achieved (see Figure 12) the expression of the Elf5 targets were analysed by real time PCR following *Elf5* knockdown. Expression was normalised to the geomean and the results were averaged and the error bars show the standard error of the mean (SEM). The expression levels are shown relative to the no treatment control, see Figure 13. For raw real time data refer to tables 16-18 in the appendices.

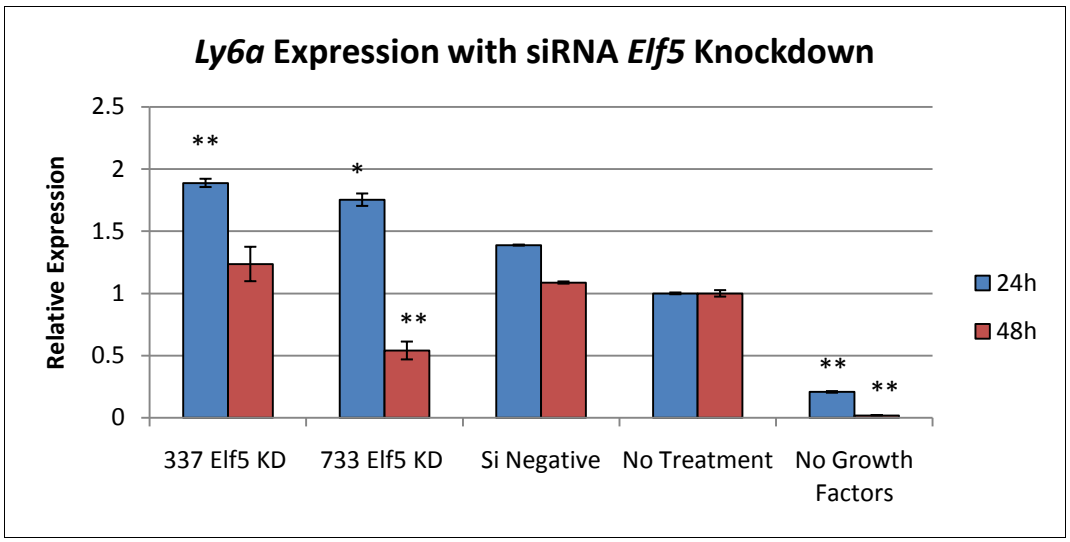


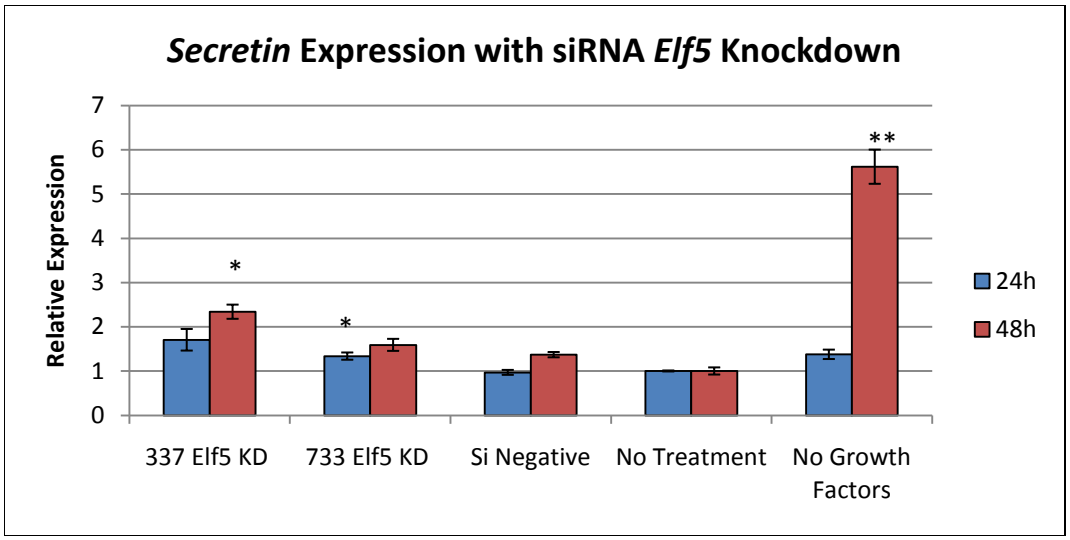


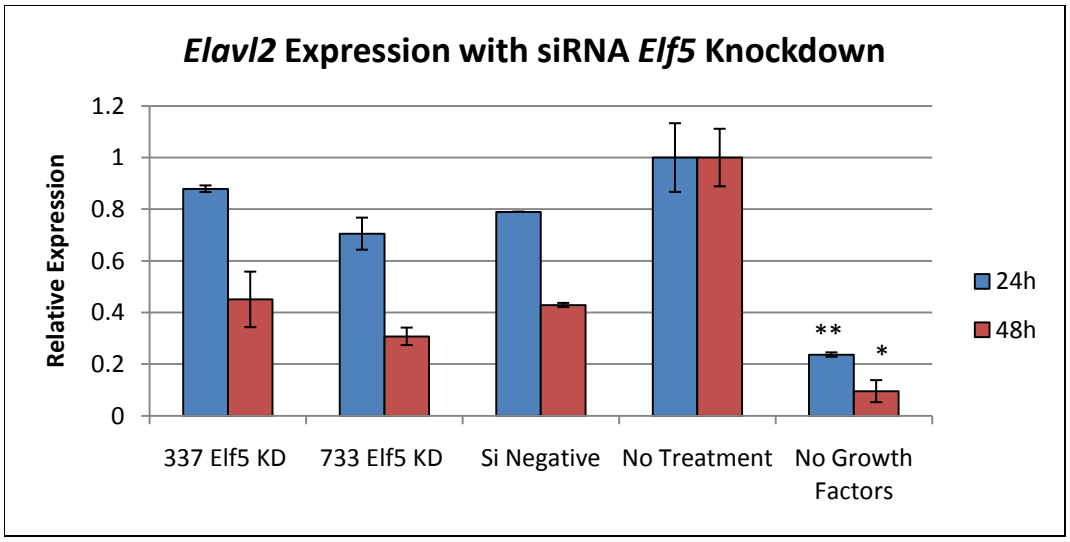

WDR40B Expression with siRNA Elf5 Knockdown
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h

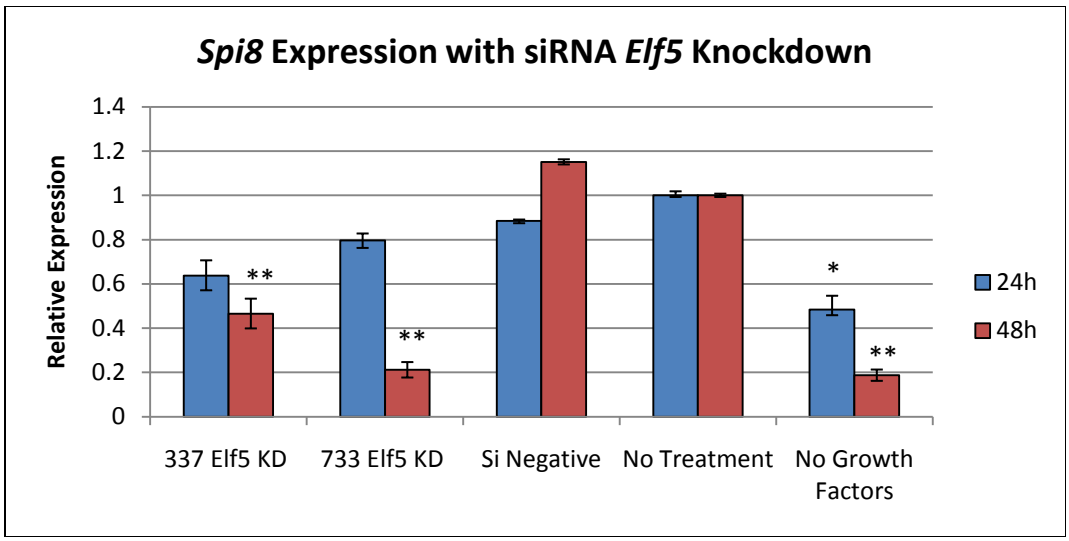

Mme Expression with siRNA Elf5 Knockdown
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h

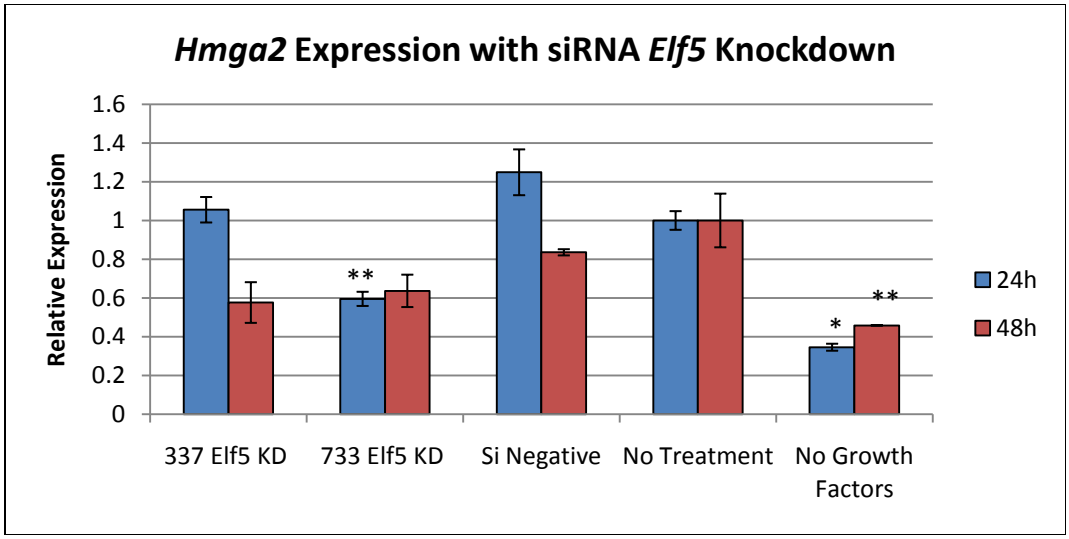

Calca Expression with SiRNA Elf5 Knockown
Relative Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h

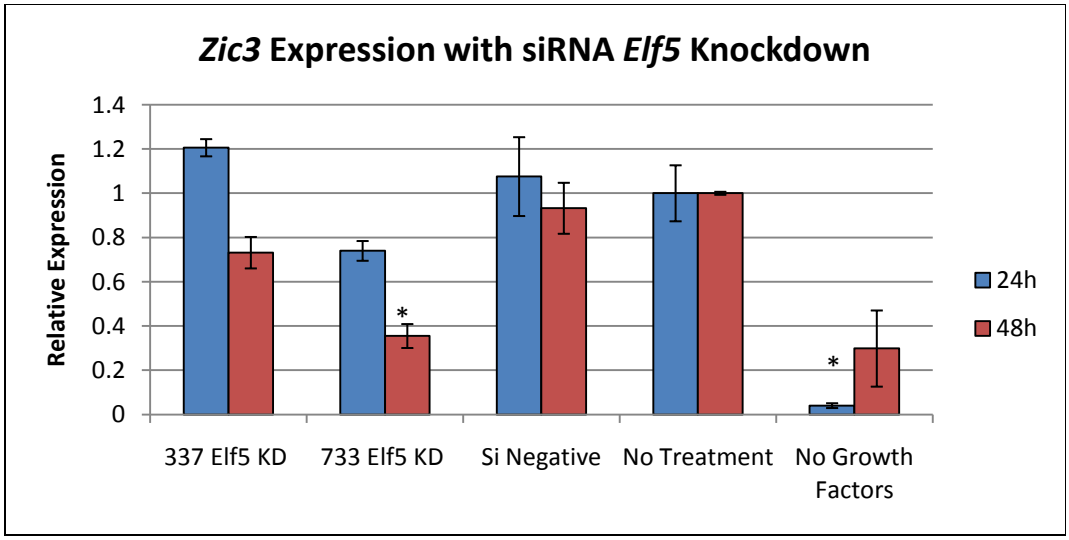

Ly6a Expression with siRNA Elf5 Knockdown
Relative Expression
2.5
2
1.5
1
0.5
0
**
*
**
**
**
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h

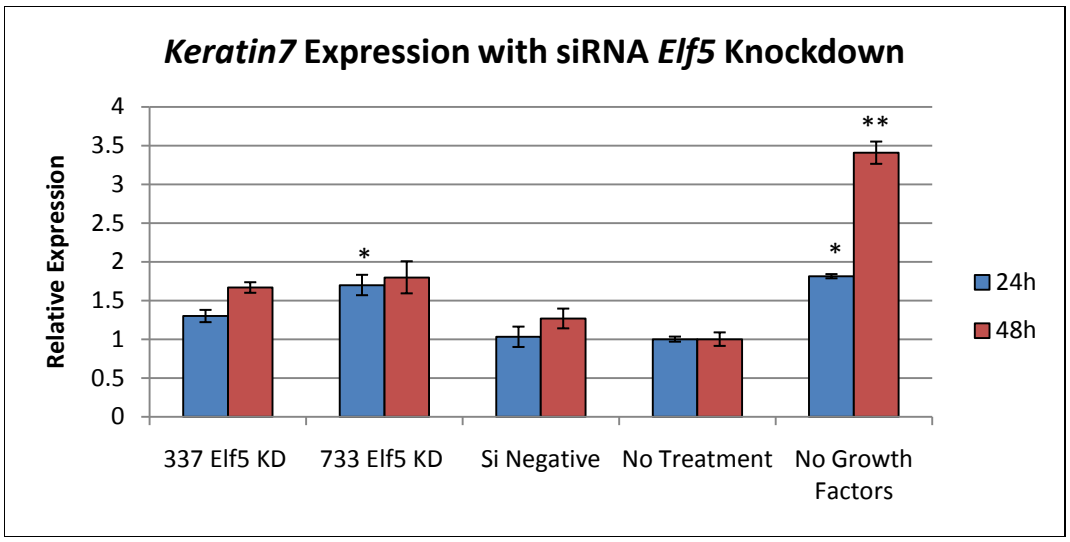

Secretin Expression with siRNA Elf5 Knockdown
Relative Expression
7
6
5
4
3
2
1
0
*
*
**
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h

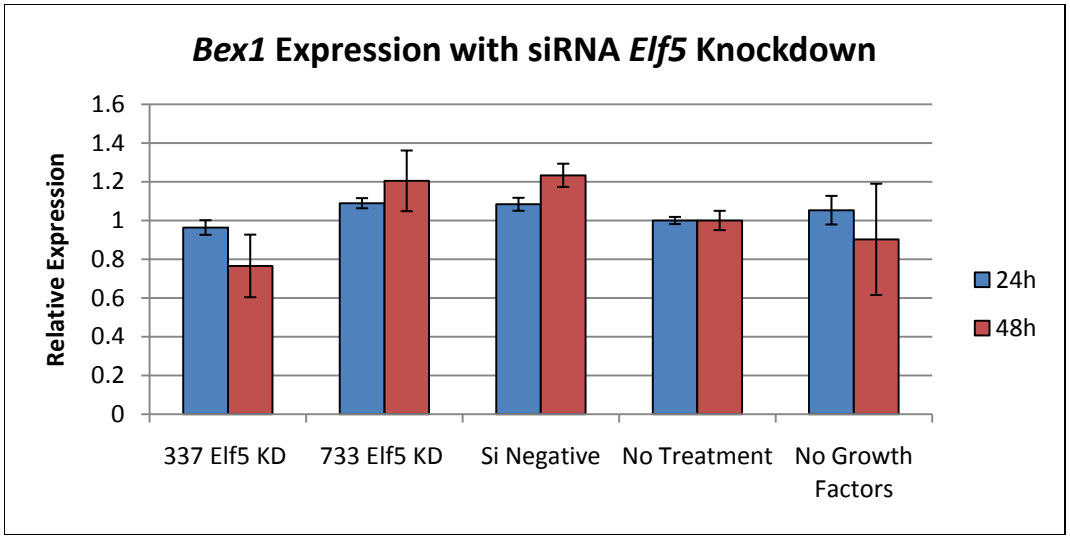

Elavl2 Expression with siRNA Elf5 Knockdown
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
**
*
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h

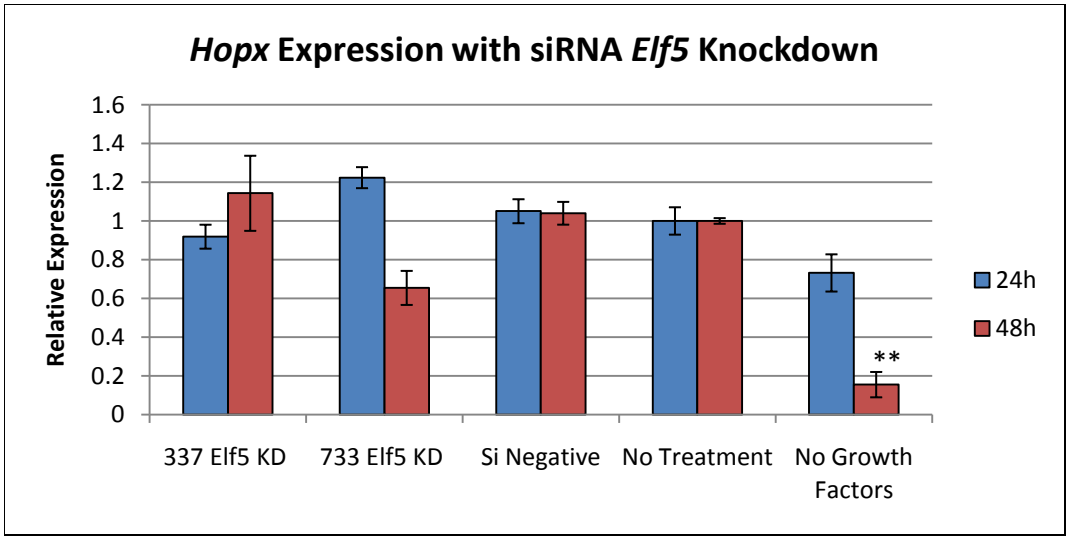

Spi8 Expression with siRNA Elf5 Knockdown
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
*
**
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h

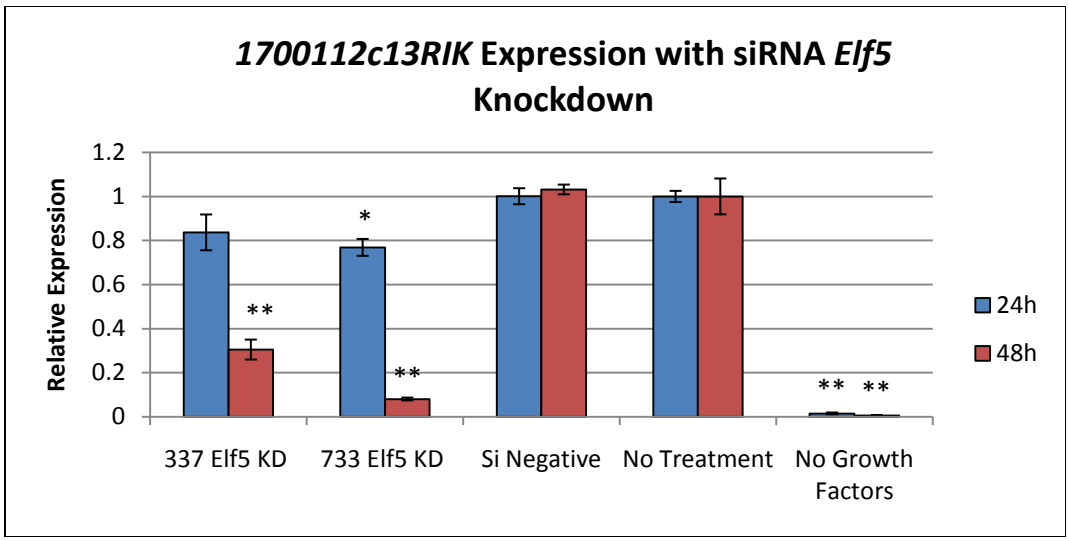

Hmga2 Expression with siRNA Elf5 Knockdown
Relative Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
**
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h


Zic3 Expression with siRNA Elf5 Knockdown
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h


Keratin7 Expression with siRNA Elf5 Knockdown
Relative Expression
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
**
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h


Bex1 Expression with siRNA Elf5 Knockdown
Relative Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h


Hopx Expression with siRNA Elf5 Knockdown
Relative Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
337 Elf5 KD
733 Elf5 KD
Si Negative
No Treatment
No Growth Factors
24h
48h



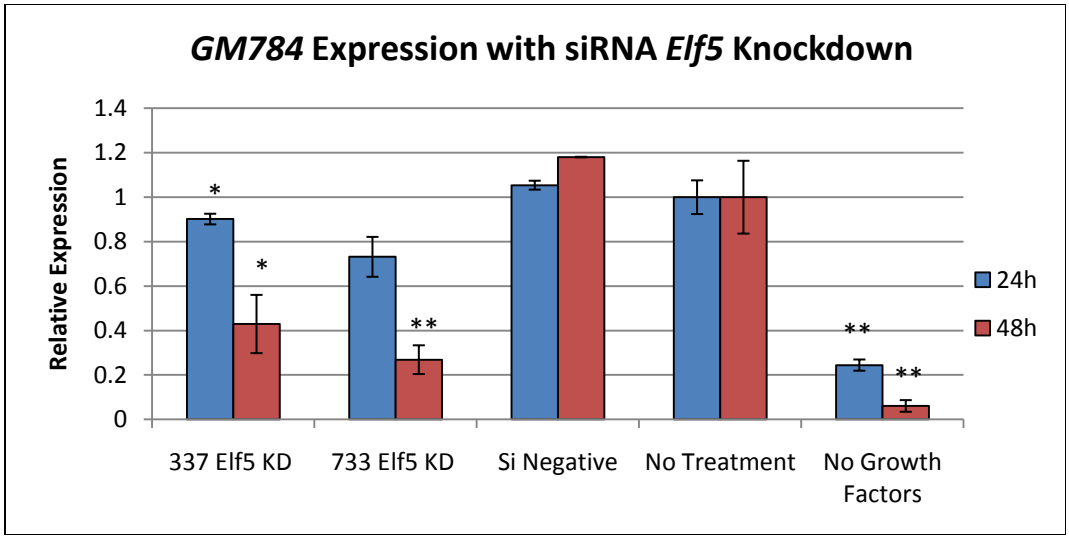


Figure 13: Changes in gene expression of the target genes in mouse trophoblast stem cells after 24 and 48 hr knockdown of *Elf5 n=3*. Expression was normalised to the geometric mean of *Gapdh, β-actin* and *β- tubulin* and then to the no treatment. Significance compared to the si negative control * p=<0.05, **p=< 0.01.

Of the 16 genes analysed 11/16 were confirmed as Elf5 target genes. These results corroborate the results from the affymetrix array carried out by Deane (2007). Suitable primers for Synpo21 could not be found. The change in expression after 24 hr was not as great as the change noted by the affymetrix array. The affymetrix results seem to lie between the 24 hr and 48 hr knockdown results. See Table 6 for a comparison of gene expression between the affymetrix array and the knockdown data obtained here. Activin was used in the TS cell culture and may have caused the subtle difference in the time it took to have an effect.

Cyr61 was rapidly down-regulated 24 and 48 hr following *Elf5* knockdown, this gene is a very promising target as the effects are rapid. It was also affected by the removal of growth factors. *Sox2* was down regulated by 20 to 40 % after 24 hr and by 70 to 80 % after 48 hr. It was dramatically affected by growth factor removal. Another promising target is *Wdr40B*. It was significantly down regulated at both time points studied and was affected just as much by growth factor removal. *Mme* was down regulated by 50 % at both time points and was affected by growth factor removal only after 48 hr. Mme may be an indirect target of *Elf5*.

Calca is another promising gene; it was significantly down regulated at both time points following the knockdown of *Elf5*. It was also severely affected by growth factor removal. There was a si negative effect in the 24 hr samples but the change in expression was still significant. *Ly6a* was difficult to interpret as the results changed. At 24 hr the gene was up regulated upon *Elf5* knockdown (as was the si negative control), the change was still significant. After 48 hr the gene seemed to be down regulated. The removal of growth factors caused the down regulation of this gene. *Secretin* was up regulated upon Elf5 knockdown and with the removal of growth factors. *Spi8* appeared to be a reproducible target, more so 48 hr after *Elf5* knockdown. It was also affected by growth factor removal. *Hmga2* was only affected by the 733 siRNA at 24 hr and then by both after 48 hr. *Zic3* follows the same trend, it was affected by only the 733 siRNA at 24 hr and then both at 48 hr. This also occurred in the microarray. *Keratin 7* was up regulated by the knockdown of *Elf5* at both time points and by the removal of growth factors. *1700112c13RIK* appeared to be a good target, it was down regulated by *Elf5* knockdown at both time points studied and was extremely affected by growth factor removal. The final gene investigated was *GM784*; it was down regulated at both time points, more so after 48 hr and was also affected in the same way by growth factor removal.

Elavl2 is not a target gene of *Elf5* as it was affected by *Elf5* knockdown in exactly the same way as the si negative control. *Bex1* is not a target of Elf5, there was no change in gene expression upon *Elf5* knockdown. There also was no change

when real time PCR was carried out on the extra microarray cDNA (the results were variable). *Hopx* is also not a target gene of Elf5 according to these results.

Table 6: Summary of *Elf5* knockdown and comparison to the Affymetrix array. Results are presented as a fold change compared to the no treatment control (N). Fold changes of the Elf5 targets were also compared to the no treatment control.

	Microarray Results					24 hr *Elf5* Knockdown					48 hr *Elf5* Knockdown				
	Up or Down	No GF vs. N	337 vs. N	733 vs. N	N vs. Si-	Up or Down	No GF vs. N	337 vs. N	733 vs. N	N vs. Si-	Up or Down	No GF vs. N	337 vs. N	733 vs. N	N vs. Si-
Elf5	-	3.4	2.4	3.1	1.0	-	2.6	4.8	6.0	1.0	-	5.1	5.5	9.3	1.0
Cyr61	-	2.0	1.3	2.0	1.2	-	1.5	2.9	2.3	1.2	-	5.9	4.9	3.5	1.4
Sox2	-	8.6	1.4	1.9	1.0	-	14.8	1.4	1.6	1.0	-	53.7	2.3	4.1	1.0
WDR40B	-	3.3	1.9	2.1	1.3 (+)	-	4.7	1.3	1.8	1.1 (+)	-	3.1	1.9	3.5	1.2 (+)
Synpo2l	-	4.2	2.1	2.4	1.1	-					-				
Mme	-	2.4	1.3	2.3	1.1	-	1.0	2.1	1.4	1.2	-	8.2	1.6	1.8	1.0
CALCB	-	2.0	1.5	1.7	1.0	-	26.3	2.0	2.4	1.4	-	95.5	2.9	6.2	1.2 (+)
Ly6a	+	5.3 (-)	1.2	2.2	1.1	+	4.8 (-)	1.9	1.8	1.4	+	51.2 (-)	1.2	1.8 (-)	1.1
Sct	+	2.2	1.5	2.2	1.2 (-)	+	1.4	1.7	1.3	1.0	+	5.6	2.3	1.6	1.4
Elavl2	-	2.1	1.0	1.8	1.1	-	4.2	1.1	1.4	1.3	-	10.5	2.2	3.3	2.3
Spi8	-	2.1	1.2	1.7	1.1	-	2.1	1.6	1.3	1.1	-	5.3	2.1	4.7	1.2 (+)
Hmga2	-	1.6	1.1	2.0	1.1	-	2.9	1.1	1.7	1.2 (+)	-	2.2	1.7	1.6	1.2
Zic3	-	7.5	1.0	2.1	1.1	-	24.8	1.2 (+)	1.4	1.1	-	3.4	1.4	2.8	1.1
KRT7	+	1.8	1.2	2.1	1.1 (-)	+	1.8	1.3	1.7	1.0	+	3.4	1.7	1.8	1.3
Rex3	-	1.3	1.4	2.1	1.1	-	1.1	1.0	1.1	1.1	-	1.1	1.3	1.2	1.2
HOPX	+	2.2 (-)	1.0	1.6	1.1	+	1.4 (-)	1.1 (-)	1.2	1.1	-	6.5	1.1	1.5	1.0
1700112C13RIK	-	3.0	1.2	2.1	1.1	-	69.3	1.2	1.3	1.0	-	199.7	3.3	12.5	1.0
GM784	-	3.3	1.6	2.5	1.2	-	4.1	1.1	1.4	1.0	-	16.5	2.3	3.7	1.2 (+)

3.3.6 Summary

Affymetrix array experiment carried out by Jessica Deane as a part of her Master's thesis, identified genes that were differentially expressed upon the knockdown of *Elf5* in mouse trophoblast stem cells. In total the gene expression of 22 genes was changed by both siRNAs. Of these, 9 were also changed by the removal of growth factors. To confirm these results the Elf5 targets of interest were analysed by repeat knockdown of *Elf5* for 24 and 48 hours and their gene expression measured by quantitative real time PCR. The expression of 16 genes were analysed in this study. These genes were from different groups identified by the affymetrix array. Those changed by both siRNAs and growth factor removal, those changed only by the knockdown of *Elf5* and those changed only by one siRNA and by growth factor removal. Interestingly, those genes that were identified as transformed by only one siRNA (733) were usually changed by both siRNAs after 48 hr of knockdown, suggesting that these genes are probably indirect targets of Elf5.

During this time the conditioned media that the TS cells were cultured in routinely was replaced by activin. Activin is thought to be one of the main components of embryonic fibroblast conditioned media and was shown by Erlebacher et al., (2004) to be able to replace conditioned media for the culture of TS cells. I found that the TS cells could be cultured in media containing activin in place of conditioned media as there was no change in gene expression apart from the up-regulation of *Cdx2* which is a TS cell marker. However, after one to two months of continuous culture in activin replacement media the morphology of the cells began to change (refer to 6.2.1.2 in chapter 6). The cells no longer grew in colonies and they had a spiked appearance, thus losing their TS cell characteristics. The gene expression at this point was not analysed. Due to this change in morphology the rest of the thesis was carried out with TS cells grown in conditioned media. The use of activin was fine for gene expression experiments or for short periods of culture but not for morphology.

4 Chapter Four: Whole mount In situ hybridisation of Elf5 targets

4.1 Overview

Whole mount *in situ* hybridization was carried out to ensure the genes of interest identified in chapter three were expressed at the right time and in the right place in the embryo *(in vivo)* to be Elf5 targets. Whole mount *in situ* hybridisation is an excellent method to get a three dimensional idea of gene expression patterns. *Elf5* is expressed in the extra embryonic ectoderm (ExE) from day 5.5 and is then later expressed in the chorion the derivative of the ExE at day 8.5, see Figure 14 below. For the genes to be targets of Elf5 signalling they must be expressed in the ExE in order to be activated or repressed by *Elf5*. If not, they are likely to be indirect targets. *In situ* hybridization allows the detection of messenger RNA in whole embryos. The ability to visualise the expression of genes in embryos is a effective device to allow the understanding of spatial and temporal relationships among different molecules during development (Irving, 2008). Non-radioactively labelled nucleic acid probes are used to visualise mRNA sequences in whole embryos. The probes are labelled with dioxygenin which are then detected with an antibody conjugated to alkaline phosphatase which converts the substrates nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) to a blue precipitate.

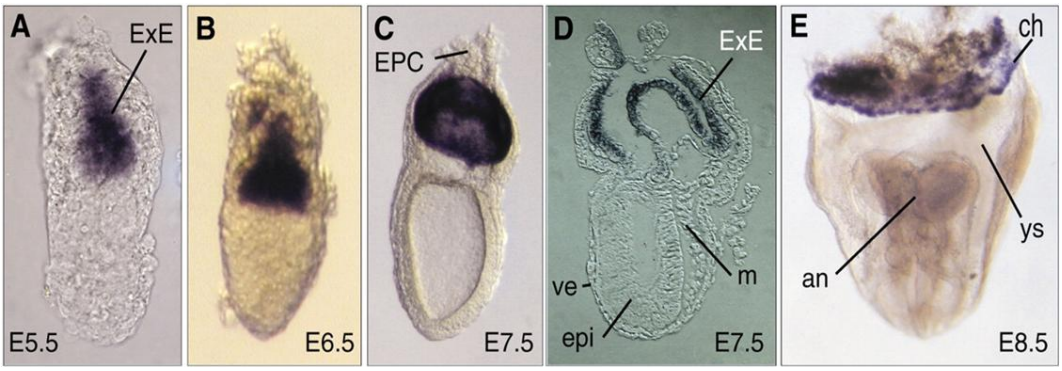


Figure 14: *Elf5* expression in the extra embryonic ectoderm (ExE) from day 5.5 to 7.5 in mouse embryos. It is expressed in the chorion at E8.5 which is the derivative of the ExE (Donnison et al., 2005). Elf5 is not expressed in the ectoplacental cone (EPC).

In chapter three the Elf5 target genes were verified by repeating the knockdown of *Elf5* in mouse trophoblast stem cells. Subsequently whole mount *in situ* hybridisation was used to verify that the targets were expressed in the right place to be activated or repressed by Elf5.

4.2 Results

4.2.1 Cloning of gene fragments to be used for Preparation of the Riboprobes

4.2.1.1 Available genes and required gene probes

Probes for Whole mount *in situ* hybridisation were required for the following genes; *Ly6a, 1700112c13RIK, Hopx, Keratin7, Elavl2* and *Zic3*. After looking at Elf5 target results from the knockdown data, probes were also made for *Calca* and *Cyr61*.

4.2.1.2 Primer design

Primers were designed according to the specifications in section 2.3.3.2. The following table contains a list of the primers used to amplify DNA for cloning.

Table 7: Primer sequences used to amplify genes of interest for probes.

Primer Name	Sequence	Length	Tm	Product length
Ly6amo-fwd2	AGTTTTATCTGTGCAGCCCTTC	21	55	612bp
ly6amo-rev2	CCTAGAGAGGATTAGAGCACC	21	55	612bp
1700112c13mo-fwd2	TGCAAGGTGGTTAAATGGGAA	21	62	875bp
1700112c13mo-rev2	AGCAGAGAGGAGGGAAAGTAG	21	62	875bp
Zic3mo-fwd2	CGAGTGCACACTGGCGAGAA	19	65	781bp
Zic3mo-rev2	AACATCACCACCACCAAGCCAT	22	65	781bp
Hopxmo-fwd2	AAGGGGACAGGGCTGAGTGAA	21	65	938bp
Hopxmo-rev2	GTGTTTAGTGAAGCAGAAGGAA	22	65	938bp
Kert7mo-fwd2	CATGCAGGATGTGGTGGAA	19	65	955bp
Kert7mo-rev2	CAGGGACATGAGATGGAACAAA	22	65	955bp
Elavl2mo-fwd2	GTACCAGTCTCCAAACAGAA	20	56	730bp
Elavl2mo-rev2	ATGTCTTCCCTTAGCTGAA	19	56	730bp
Cyr61mo-fwd2	GAAGAGTGGGTTTGTGATGAA	21	55	765bp
Cyr61mo-rev2	AAGAATGAGCAAGGCACCAT	20	55	765bp
Calcamo-fwd2	AAGAGTCACCGCTTCGCA	18	55	813bp
Calcamo-rev2	AGAAGAGCAAGAGGCAGTGT	20	55	813bp

Cloning using PCR

The genes of interest were cloned from cDNA from previous experiments. The cDNA used was from mTS cells grown in complete medium for samples that are down regulated upon the loss of *Elf5* (*1700112c13RIK, Elavl2, Zic3, Calca and Cyr61*) and TS cells grown in mTS media with no growth factors for those genes that are up regulated upon the loss of *Elf5* (*Ly6a, Hopx, Keratin 7*). A PCR was carried out to amplify the genes of interest, see 2.3.3.3 and then the resulting DNA was run on a 1 % agarose gel, see Figure 15 below.

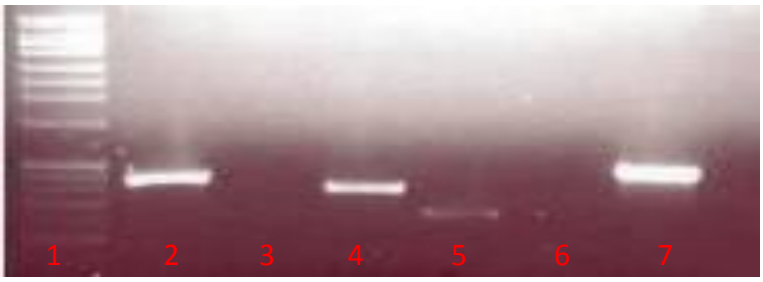


Figure 15: amplification of genes of interest by PCR. 1=1kb ladder, 2= *1700112c13* product, 3= *Elavl2* product, 4=*Zic3* product, 5= *Ly6a* product, 6= *Hopx* product, 7= *Keratin7* product. No product was obtained for *Elavl2, Ly6a or Hopx*.

4.2.1.2.1 *Trouble shooting 1:*

No Products were obtained for *Elavl2* and *Hopx* and only a faint band for *Ly6a,* see Figure 15. This was probably due to inappropriate annealing temperature for the specific primer. So a temperature gradient from 54 °C to 64 °C was carried out to find the optimum annealing temperature. *Ly6a* worked at all temperatures tested. *Elavl2* only worked at 54 °C and *Hopx* didn't work at all (Figure 16).

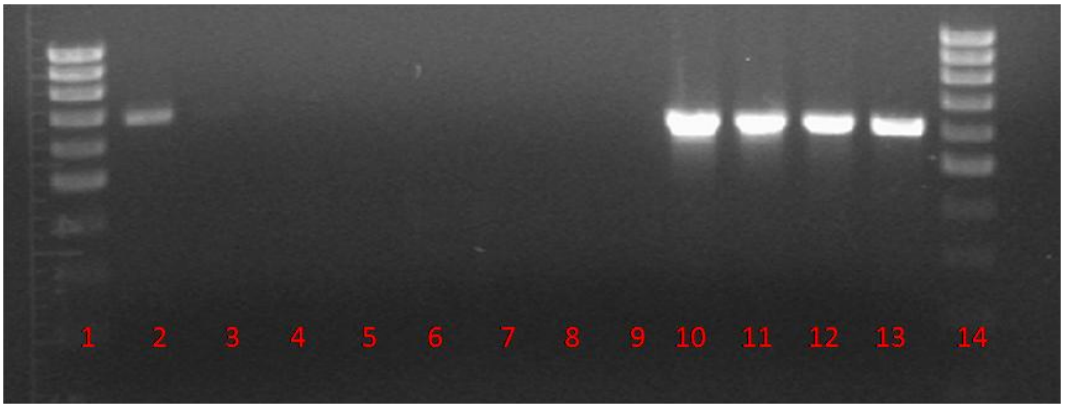


Figure 16: PCR gradient results to check primers. 1= 100bp ladder, 2-5 *Elavl2*, 6-9 *Hopx*, 10-13 *Ly6a*, 14= 100bp ladder. Ly6a worked at all temperatures, Elavl2 worked at 54 °C and Hopx did not work.

4.2.1.2.2 Trouble shooting 2:

As *Hopx* is a GC rich gene it was suggested to try a 5x GC-rich solution in the PCR reaction. When a gene is quite GC rich the polymerase tends to fall off the DNA. The temperature gradient was carried out again, and a product of the correct size was obtained for an annealing temperature of 54 °C, Figure 17.

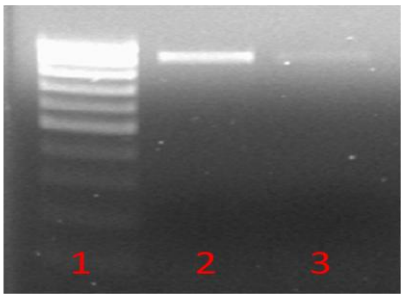


Figure 17: Optimised PCR conditions for Hopx, 1 = ladder, 2 is *Hopx* at 54 °C, 3 is *Hopx* at 56.8 °C.

Once the optimal PCR conditions were determined, all of the products were re-amplified using the optimal conditions.

4.2.1.3 Purification of the PCR product

The PCR product was purified with a Wizard column as described in 2.3.2.4.1

4.2.1.4 Ligation of PCR product into pGEM-Teasy

The PCR product was ligated into the pGEM- T Easy plasmid (Promega) so it could then be transformed into *E. coli* to be amplified. The pGEM-T Easy vector contains a single T overhang at the insertion site to increase the efficiency of

ligation of the PCR product into the plasmid. This is by preventing re-circularisation of the plasmid and providing T overhangs for the A overhangs of the insert produced by the *Taq* polymerase. pGEM-T Easy contains T7 and SP6 RNA polymerase promoters flanking the multiple cloning site (MCS) in the coding region of the protein β galactosidase, see Figure 18. When the PCR product is inserted into the MCS this protein is no longer expressed and *E. coli* colonies containing the insert can be selected by the colour of the colony (Promega, 2007).

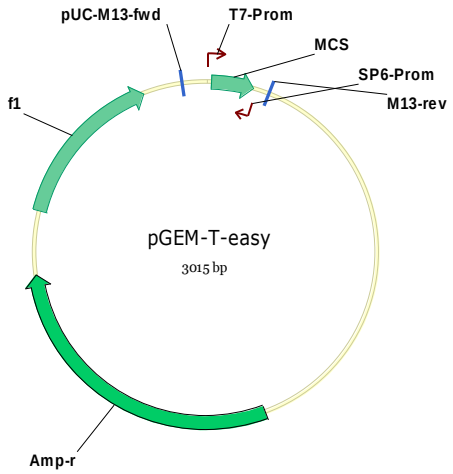


Figure 18: Diagram of pGEM-T-easy used to ligate the DNA of interest into and to transform into *E. coli.* There was an ampicillin resistance gene so only cells that had the transformed plasmid could grow on the LB/Amp plates. The DNA was ligated into the multiple cloning site (MCS).

4.2.1.5 Transformation

The plasmid containing the PCR product was transformed into Invitrogen Max Efficiency® DH5α™ Competent Cells to amplify the plasmid containing the DNA of interest. The method for the transformation of the plasmid into *E. coli* is described in 2.3.2.6.

4.2.1.6 Minipreps

Minipreps are carried out to isolate the amplified plasmid from the bacterial culture. The miniprep method is described in 2.3.2.7. Only 6 colonies of each gene were minipreped as TA cloning is efficient and blue/white selection was used.

4.2.1.7 Restriction analysis

3 µl was analysed by restriction digest with EcoR1 to make sure the clones contained the insert. The DNA as digested over night at 37 °C. There are EcoR1 sites on either side of the MCS (see Figure 19); the product obtained should be the same size as the product produced from the primers for the gene of interest.

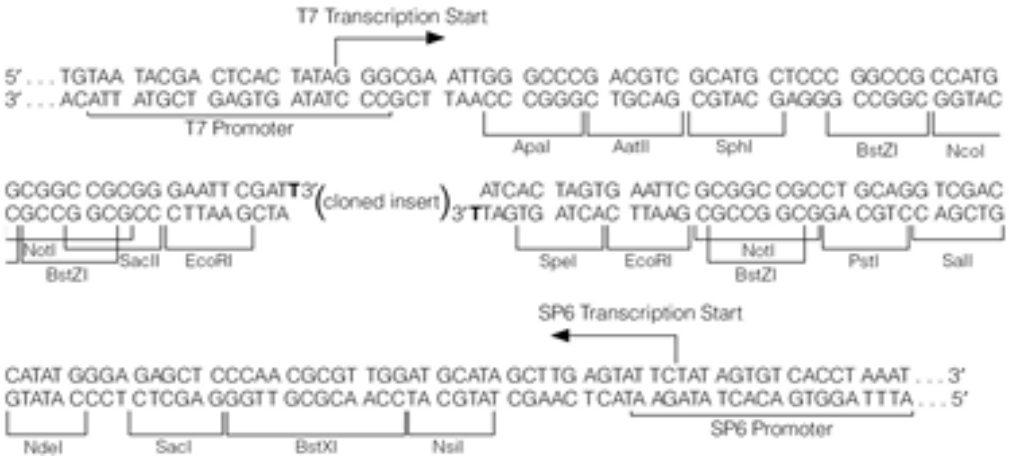


Figure 19: Multiple cloning site (MCS) of the pGEM-T Easy plasmid showing EcoR1 restriction sites on either side of where the gene of interest DNA was inserted.

The digest was then run on a 1 % agarose gel against a 1 Kb ladder to determine which clones contained the insert of interest. The *Keratin 7* and *Zic3* products had internal EcoR1 sites so two products would be produced instead of one. The expected product sizes were; *1700112c13*- 875 bp, *Elavl2*- 730 bp, *Ly6a*- 612 bp, *Keratin7*- 189 bp and 745 bp, *Zic3*- 635 bp and 124 bp (see figures 20-22).

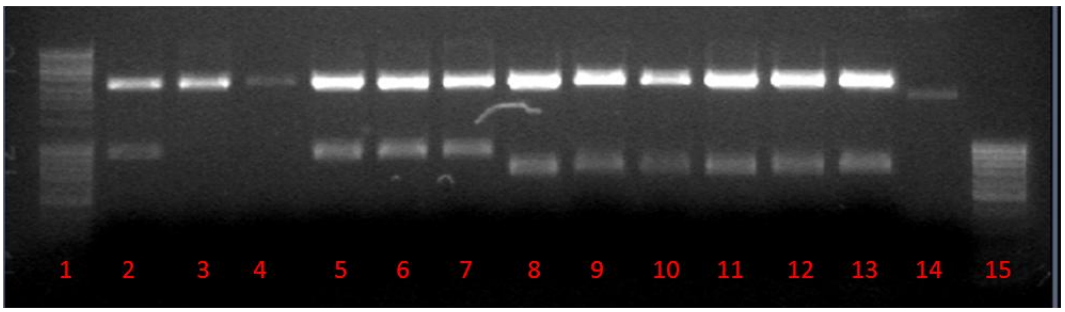


Figure 20: Restriction digests of the miniprep DNA to ensure a product of the expected size was obtained. 1 = 100 Kb ladder, 2-7 = *1700112c13* clones, 8-14 = *Elavl2* clones, 15= 1 kb ladder. Products of the expected size were obtained.

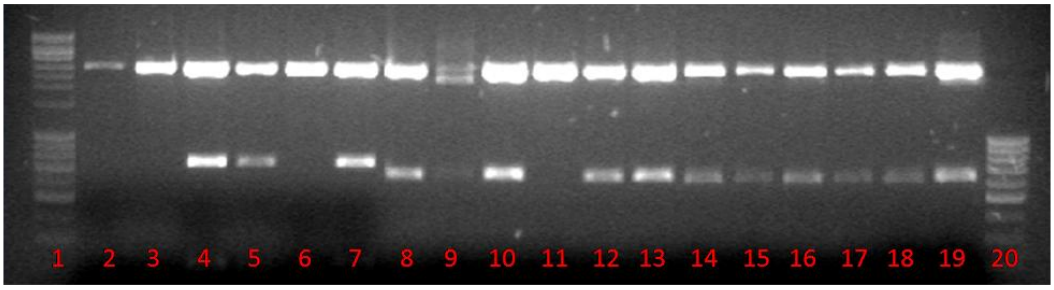


Figure 21: Restriction digest of the miniprep DNA to ensure a product of the expected size was obtained. 1= 100 kb ladder, 2-7 = *Keratin7* clones, 8-14 = *Zic3* clones, 15-19= *Ly6a* clones, 20 = 1 kb ladder (can't see smaller product of *Keratin 7* and *Zic3* as they are only about 100 bp). Products of the expected size were obtained.

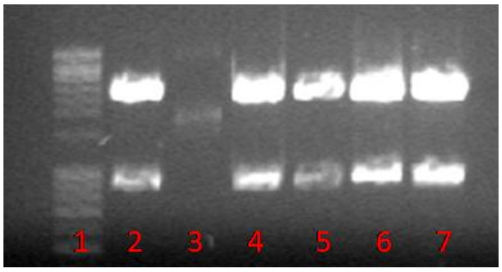


Figure 22: 1= 100 kb ladder, 2-7 = *Hopx* clones of the correct size.

4.2.1.8 Determination of orientation of the product

Restriction digest was then carried out with the clones that had the correct sized insert to determine the orientation of the PCR product. As TA cloning was used the PCR fragment could be inserted in the forward or reverse orientation. The genes of interest were analysed on Vector NTI to determine what restriction enzymes could be used to produce two fragments of different size to determine what orientation the genes were in. The restriction site should be present in the PCR fragment and in the MCS to get appropriate sized products. Table 8 shows what restriction enzymes were used to determine the orientation of the products.

Table 8: Restriction enzyme used to determine orientation of each insert in p-Gem-Teasy

		Product size (bp)	
Gene	RE	Forward	Reverse
Ly6a	Pst1	197	420
1700112c13	Pst1	768	85
Hopx	Pst1	787	128
Keratin 7	Sac1	389	677
Elavl2	Sac1	340	483
Zic3	Clal 1 and Pst1	23	757

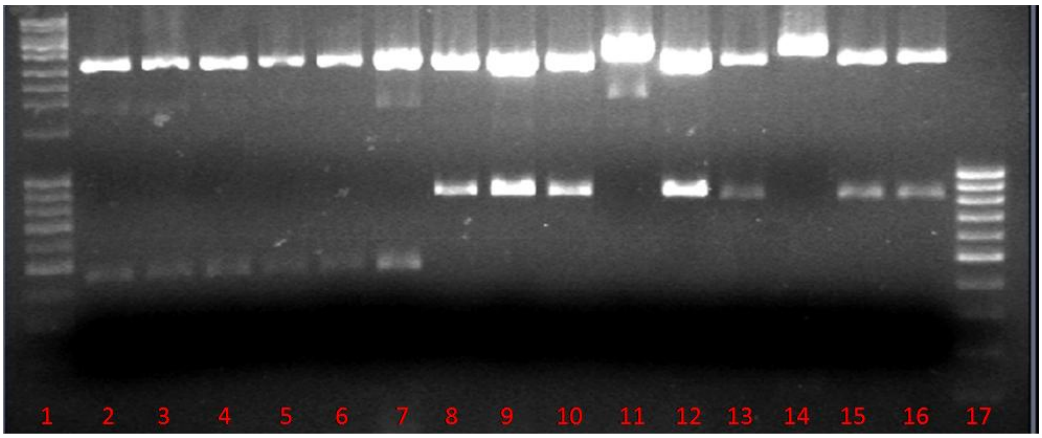


Figure 23: 1 = 100 kb ladder, 2-7 = *Ly6a* clones, 7- 12 = *Hopx* clones, 13- 16 = *1700112c13* clones, 17 = 1 kb ladder. All the *Ly6a* clones had their insert in the reverse orientation, *Hopx* clones were in the forward orientation. *1700112c13* clones were in the forward orientation.

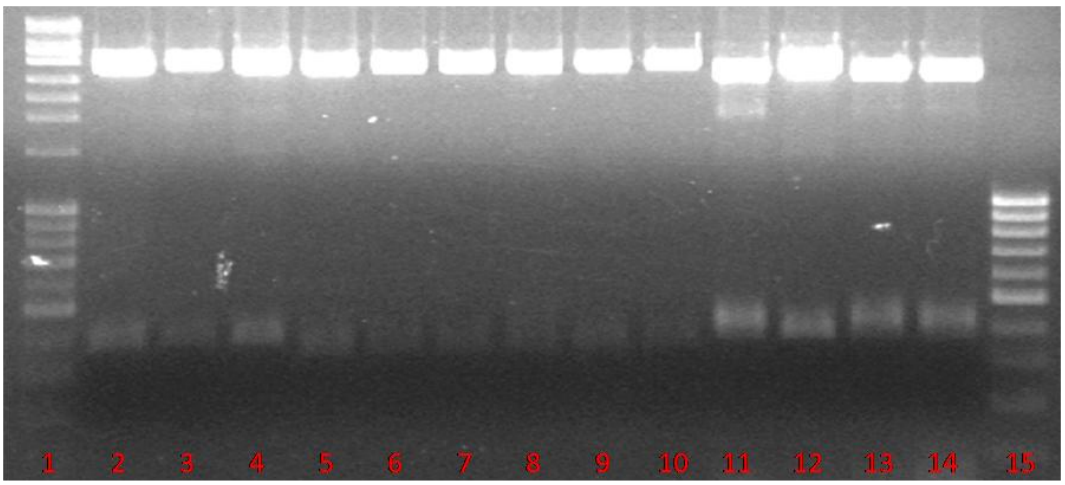


Figure 24: 1 = ladder, 2-4 = *Keratin7* clones, 5- 10 = *Elavl2* clones, 11 – 14 = *Zic3* clones,15 = 1 kb ladder. *Keratin7* was in the forward orientation, *Elavl2* forward and *Zic3* reverse.

4.2.1.9 Glycerol Stocks

One clone showing the correct restriction analysis for each gene was used to make a glycerol stock see 2.3.3.10. The stocks were flash frozen by immersion in liquid nitrogen for a few seconds, and then stored at -80°C until required.

4.2.1.10 Maxi Preparation of DNA

A maxiprep was then carried out of one colony of each gene showing the correct sized fragment and orientation see 2.3.3.11. The DNA concentration from the maxi prep was measured on the nanodrop.

Table 9: Nanodrop measurements of DNA concentration and purity

Sample	ng/ul	260/280	260/230
Hopx	982.8	1.91	2.31
Keratin 7	4966.6	1.17	1.41
Zic3	4998.2	1.08	1.3
Elavl2	5020	1.07	1.28
1700112c13	1888.8	1.88	2.29
ly6a	2951	1.84	2.25

I was concerned as the 260/280 and 260/230 ratio for *Keratin 7, Zic3* and *Elavl2* were low, see Table 9. A 260/280 ratio of 1.8 or greater is considered pure for DNA; a lower ratio could have been due to phenol, protein or other contaminants that absorb strongly at 280 nm. A 260/230 ratio measures nucleic acid purity, a pure sample has a ratio of 2.0-2.2. It was suggested that the values for the three samples were out of the linear range of the nanodrop as the DNA concentration was very high. So the DNA was diluted 1:10 in T.1E and the DNA concentration was measured again.

Table 10: Nanodrop measurements after dilution of the DNA

1:10 Dilution	ng/ul	260/280	260/230
Keratin 7	929.6	1.91	2.31
Zic3	1098.6	1.91	2.33
Elavl2	954.6	1.92	2.33

The ratios improved considerably and were now in the acceptable range, Table 10. The DNA of these three samples was diluted 1:10 for further use.

4.2.1.11 Sequencing

Aliquots of maxiprep DNA were sequenced at the Waikato University DNA sequencing facility. This was to ensure the ligation sites were correct. Sequencing primers were either SP6 (reverse orientation) or T7 (forward orientation) polymerases. The sequence of the insert could then be compared to that of the original by using vector NTi software. Any mismatches were checked if they were real by inspecting at the chromatogram to see if an incorrect call had been made by the software.

4.2.1.12 Probe Template Preparation

To prepare a RNA probe, the template must be first linearised by restriction enzymes. Restriction enzymes to use were determined by finding an enzyme that cuts uniquely on the 5' end and that creates a 5' overhang. If the insert was in the forward orientation in the plasmid then the SP6 RNA polymerase was used to create an antisense RNA copy of the DNA. And if in the opposite orientation the T7 RNA polymerase was used. The Table 11 below shows what restriction enzymes were used and what polymerase to make an RNA copy of the DNA.

Table 11: Enzymes used to linearise the DNA for probe template preparation

Sample	RE	Polymerase
Elavl2	Asp718	Sp6
Hopx	NcoI	Sp6
Keratin 7	SphI	Sp6
Ly6a	Sal1	T7
Zic3	Sal1	T7
1700112c13	NcoI	Sp6

The DNA was linearised by following the procedure in 2.3.3.13. The concentration of the DNA was estimated by running a 1:10 dilution of the DNA on a 1 % gel against 2 µl and 5 µl of a lambda standard, Figure 25.

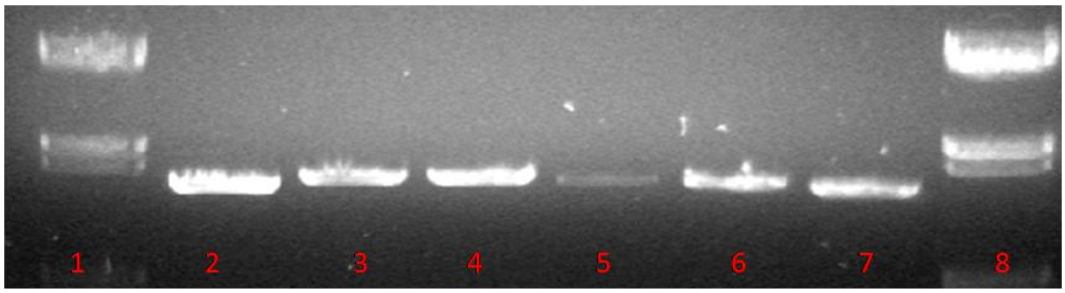


Figure 25: 1 = 2 µl of Lambda DNA 2 = *Elavl2* DNA 3 = *Hopx* DNA 4 = *Keratin 7* 5 = *1700112c13* 6 = *Ly6a* 8 = 5 µl of Lambda DNA standard. The DNA concentration of each sample was estimated by the intensity of the DNA band in comparison to the lambda DNA ladder.

1700112c13 had a low DNA concentration so the lineraisation and purification was repeated which resulted in a higher yield. The DNA was then used to prepare DIG probes.

4.2.1.13 Preparation of Riboprobes

The riboprobes were prepared by following the protocol in 2.3.3.14. After 2-3 hours the RNA was run on a 1.4 % gel with a Lambda DNA ladder. A thin sharp slow migrating DNA band was expected along with two intense RNA bands that were smaller in molecular weight. If bands were what was expected they were run through a Roche quick spin column for RNA. This column works by size

exclusion, where the larger molecules will be eluted at an earlier time than the smaller molecules as they get absorbed into the matrix. This allows the separation of nucleic acids from other molecules like the labelling mix. The RNA probe was then ethanol precipitated, washed and the pellet was resuspended in DEPC water. The RNA concentration of the probe was estimated by using the nanodrop (see Table 12) and then run on a 1.4 % agarose gel (see Figure 26).

Table 12: RNA concentration and quality of the riboprobes

Sample	ng/ul	260/280	260/230
Hopx	534.9	1.85	1.93
Keratin 7	543.9	1.82	1.8
Zic3	507.7	1.87	1.92
Elavl2	37.7	1.94	1.53
1700112c13	522.6	1.82	1.92
ly6a	516.6	1.9	1.97

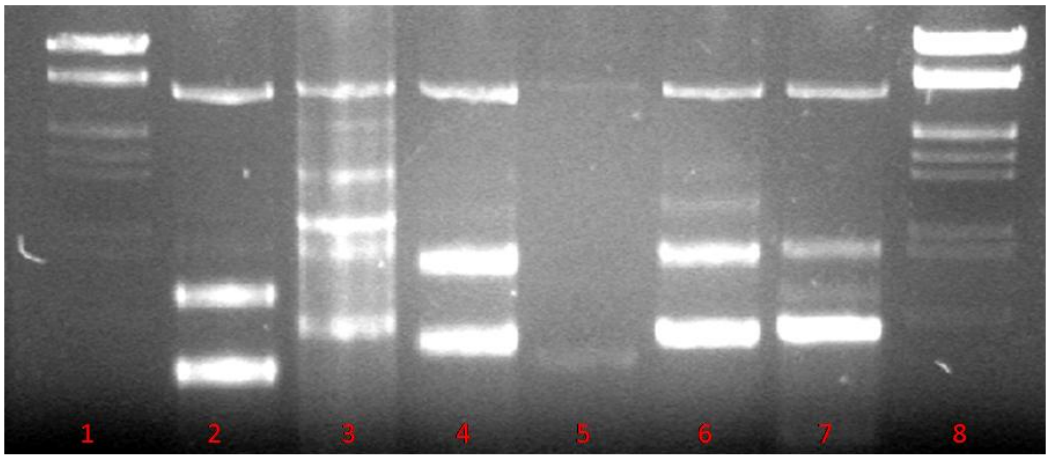


Figure 26: Gel of the riboprobes. 1 = 2 µl Lambda DNA ladder, 2 =*Ly6a,* 3 = *Keratin 7,* 4 = *Zic3,* 5 = *Elavl2,* 7 = *1700112c13,* 8 = 5 µl Lambda ladder. The *Elavl2* probe was lost and the *Keratin 7* probe ran oddly.

4.2.1.13.1 Trouble shooting (Figure 26)

The *Elavl2* pellet was lost presumably by the aspiration after the precipitation step and the *Keratin 7* probe (lane 3) ran oddly on the gel (see above gel). This could have been due to degradation or due to incomplete digestion with the restriction enzyme. Preparation of the riboprobe was repeated for *Elavl2* and *Keratin 7*. A probe of good concentration was obtained for *Elavl2* with an RNA concentration of 478.4 ng/µl and a 260/280 ration of 2.08 and 260/230 ratio of 2.04. After RNA was transcribed for *Keratin 7*, it was run on a 1.4 % gel. The same

thing occurred, the smear of RNA reaching to very high molecular weight suggested read through with random termination which would happen if the DNA had not been cut properly with the restriction enzymes. So the *Keratin 7* DNA was instead cut with the restriction enzyme Asp718 and the probe was re-transcribed. A good RNA probe was obtained with a concentration of 439.8 ng/µl.

4.2.2 Whole mount *in situ* hybridisation

Elf5 is expressed in the embryo between the developmental stages of E5.5 to E8.5 (see Figure 14). Whole mount *in situ* hybridisation was carried out to ensure the genes of interest were expressed in similar spatial and temporal patterns as *Elf5*. E5.5 embryos are extremely difficult to obtain due to their very small size and E8.5 embryos show more background staining (P. Pfeffer, personal communication). Due to this we decided to focus on E7.5 embryos and then once I was more experienced we also included the E6.5 embryos.

The embryos were retrieved from pregnant mice after timed matings from the small animal colony at AgResearch, Ruakura. See section 2.3.4.1 of methods. The protocol was followed in 2.3.4.2-2.3.4.5 to give successful production of a blue precipitate where the mRNA of the gene of interest was expressed.

4.2.2.1 Pilot experiment one:

There was no colour development observed for *Hopx* and *Elavl2* after three days in the BCIP/NBT colour development solution. *Zic3* developed at the epiblast end of the embryo at days 6.5 and 7.5. *Ly6a* seemed like the best probe, developing in the ExE of day 7.5 and day 6.5 embryos. *Keratin 7* looked like it was expressed in the EPC of the embryos, but needed further embryos for conformation. 1700112c13 was expressed in the chorion of day 7.5 embryos and the tip of day 6.5 embryos. There were problems with precipitate forming all over the embryos; this was thought to be due to old $MgCl_2$ in the NTMT. This resulted in a lot of embryos that couldn't be used as they had a black precipitate all over them, when a photograph was taken the black precipitate could not be

distinguished from the DIG probe. All components of the NTMT were made up fresh for the next time.

4.2.2.2 Pilot experiment two:

Only day 7.5 embryos were used. The probes were diluted 1:5 in hybridisation mix to allow longer storage at -80 °C. As we were getting a problem with precipitation of the NTMT the embryos were put in solutions made from NBT/BCIP tablets. No colour development was seen after 3 days incubation at room temperature. This could have been due to many reasons including, probe degradation, incorrect salt concentration in the hybridisation mix, the secondary antibody could have gone off, the Tris may have not been the correct pH and the tablets were expired. The pH of the Tris buffer was checked, it was the correct pH (pH 9.5) so that was not the reason and the salt concentration was correct in the hybridisation mix. Then 5 µl of the probes were run on a 1.4 % gel to ensure they had not been degraded by RNases. The RNA probes were not degraded. It was concluded that the problem was either the secondary antibody or the NBT/BCIP tablets. I decided to try adding the secondary antibody again to see if any colour develops, the embryos were washed 3 x in MABT and then the protocol was followed from the blocking step (refer to 2.3.4.4). Some colour developed after a few days in freshly made NTMT with BCIP/NBT but not with the tablets. These tablets were not used for subsequent experiments.

4.2.2.3 *In vivo* expression

Zic3 was expressed in the epiblast at E6.5 and then the embryonic mesoderm or ectoderm at E7.5. *Keratin 7* was expressed at low levels in the visceral endoderm on the anterior side and in the giant cells/EPC at E7.5 and in the EPC at E6.5; the expression in the ExE was background. It was expressed in late embryos too. *1700112c13* was expressed strongly in the chorion at late day 7.5 embryos. But there was no staining in earlier embryos. *Ly6a* was expressed in the ExE at day 6.5 and the chorion at day 7.5. *Elavl2* was expressed in the ExE in both E6.5 and E7.5 embryos. See Figure 27and Figure 28 for a schematic of the different tissues of the embryo at these early stages. Figure 27 shows a stage earlier than Figure 28.

After deciding what genes look interesting I also made probes for *Calca* and *Cyr61*, the same protocol was followed and successful results were obtained. *Calca* was expressed in the node of E7.5 embryos. *Cyr61* was expressed in the ExE at E6.5 and later the chorion and allantois at E7.5.

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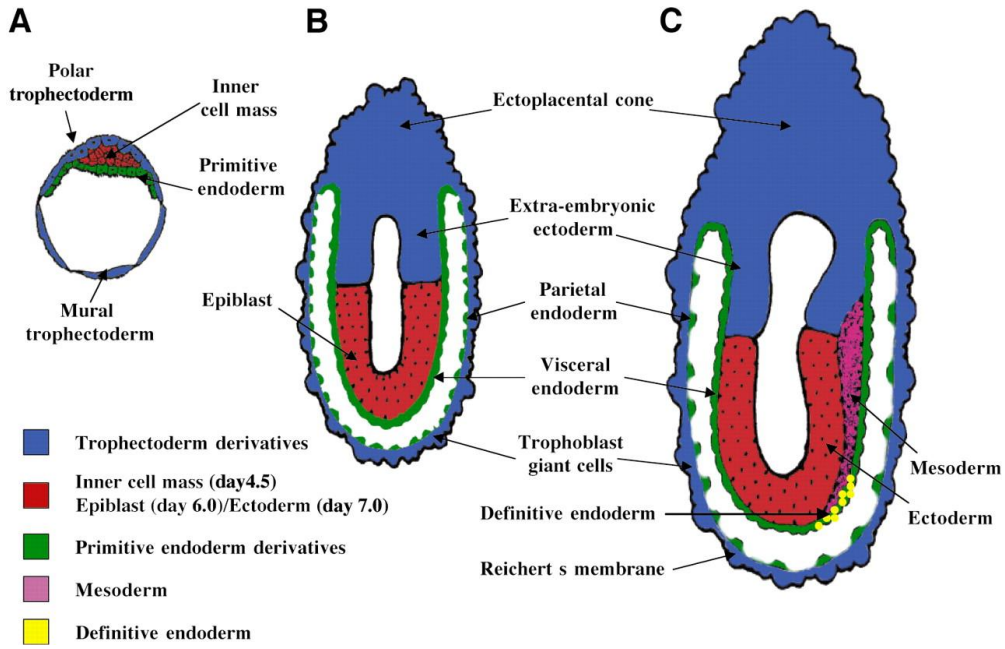


Figure 27: Mouse embryos at the periimplantation (A, E4.5) and post-implantation (B, E 6.0; C, E 7.0) stages of development, showing the allocation of derivatives of the inner cell mass/epiblast, primitive endoderm and trophectoderm, to different tissue compartments of the embryo (Tam and Rossant, 2003).

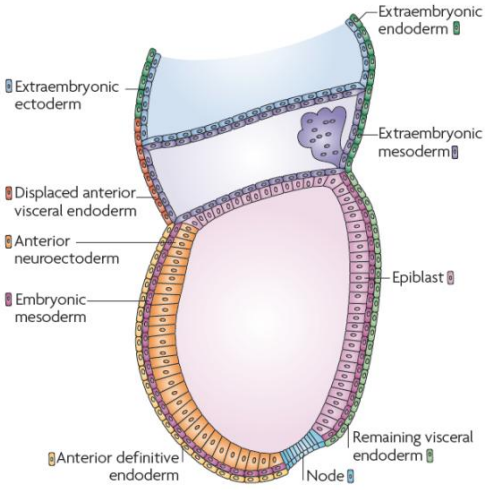


Figure 28: Figure of different cell types at gastrulation from Arnold & Robertson (2009). This is a later stage embryo, E7.5. This figure shows the Node of the embryo where *Calca* was expressed.

4.2.2.4 *In vivo* Expression of Elf5 targets in E6.5 embryos

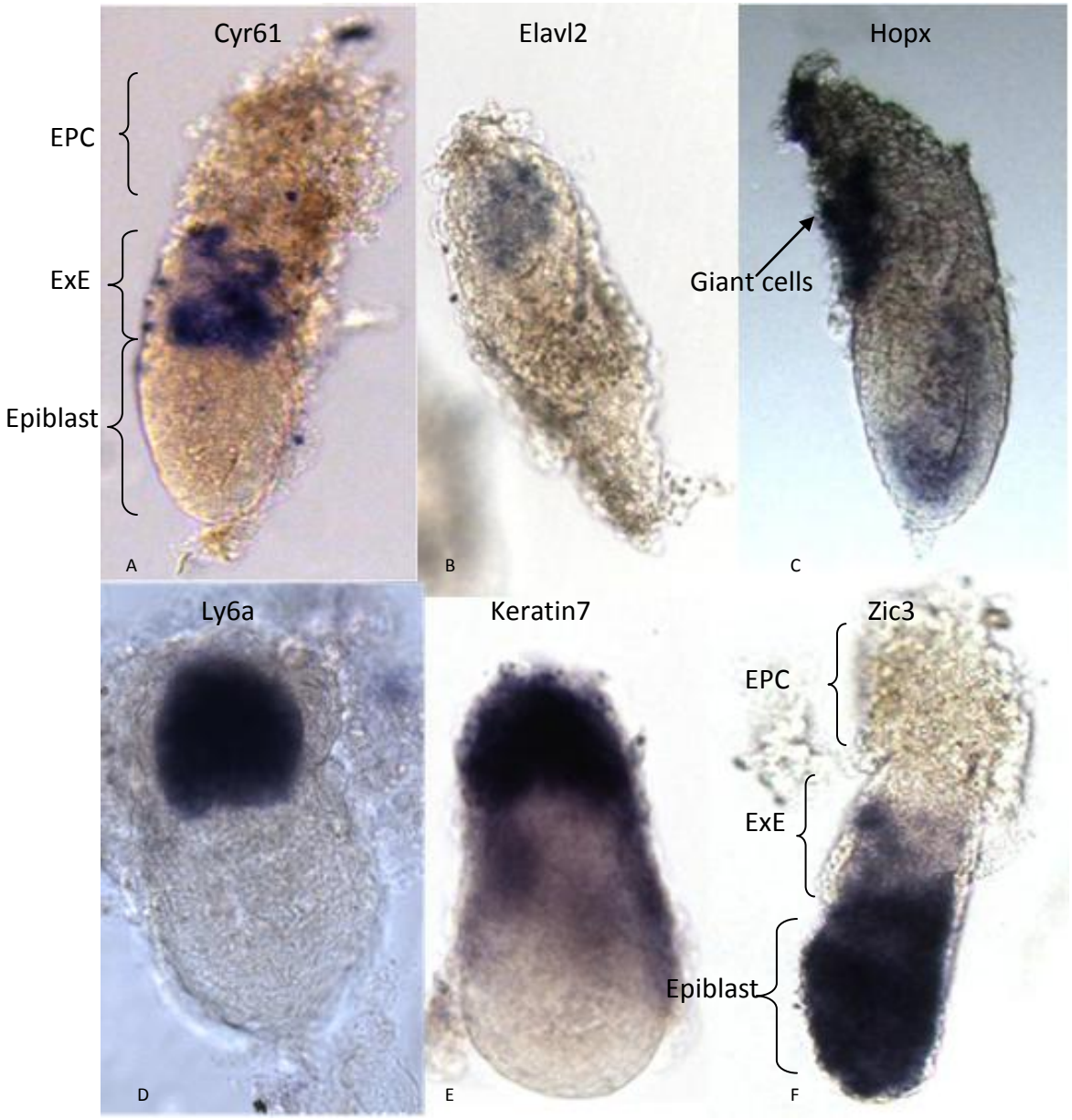


Figure 29: Expression of genes of interest at E6.5. A) *Cyr61* expressed in the ExE B) *Elavl2* expressed in the ExE C) *Hopx* expressed in giant cells D) *Ly6a* expressed in the ExE E) *Keratin7* expressed in the EPC F) *Zic3* expressed in the epiblast. *Calca* could not be detected at E6.5 and neither was *1700112C13.*

4.2.2.5 *In vivo* expression of Elf5 targets in E7.5 embryos

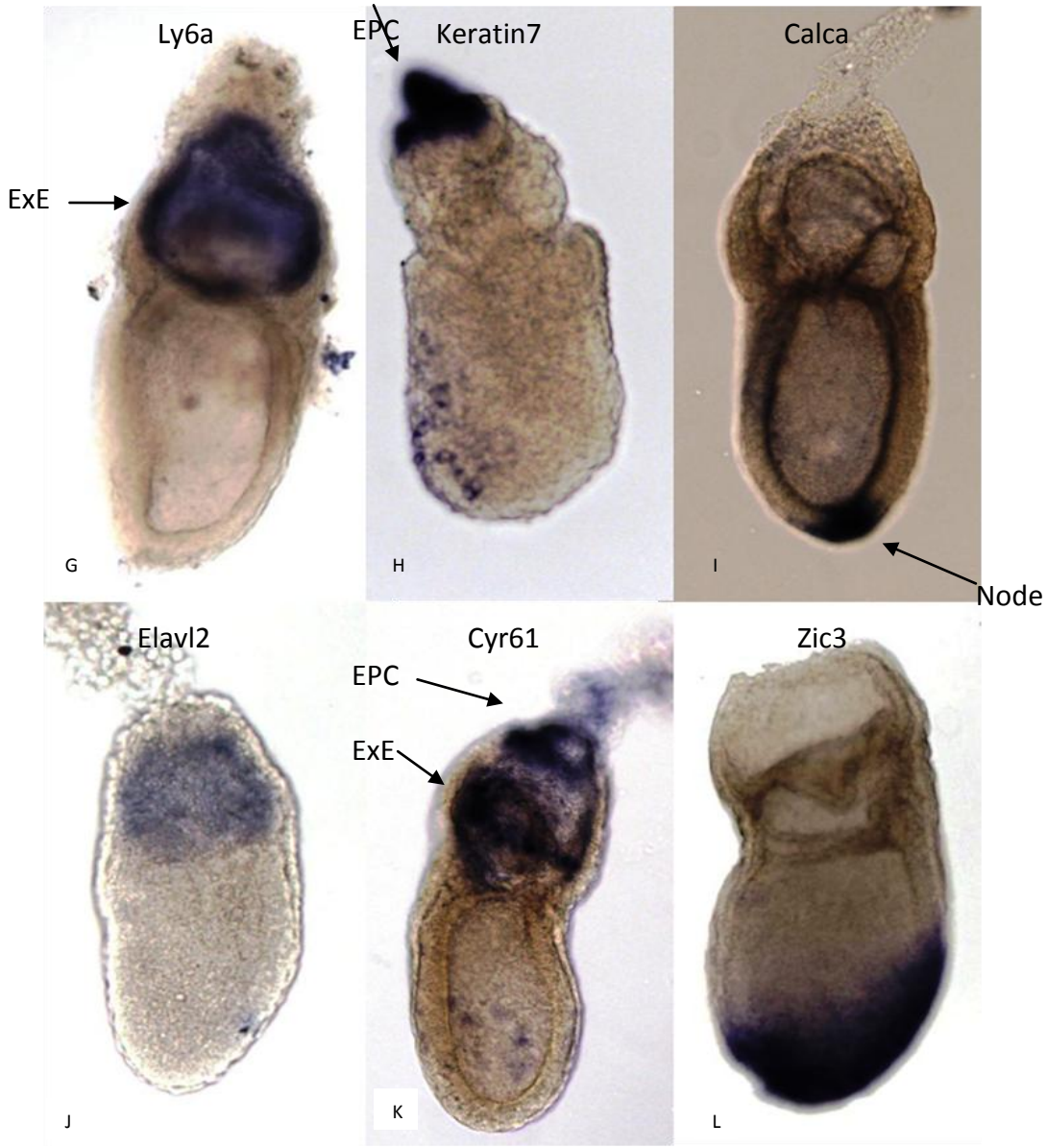


Figure 30: Expression of genes of interest at E7.5. G) *Ly6a* was expressed in the ExE H) *Keratin 7* expressed in the EPC I) *Calca* expressed in the node J) *Elavl2* expressed in the ExE K) *Cyr61* expressed in the ExE L) *Zic3* expressed in the embryonic mesoderm or ectoderm. *Hopx* has not been included as only stains giant cells surrounding the embryo. *1700112c13* was expressed at in late D7.5-8.0 embryos unfortunately no good photographs were able to be obtained.

4.2.2.6 Other target genes

The expression of other possible Elf5 targets *in vivo* were examined by Martyn Donnison.

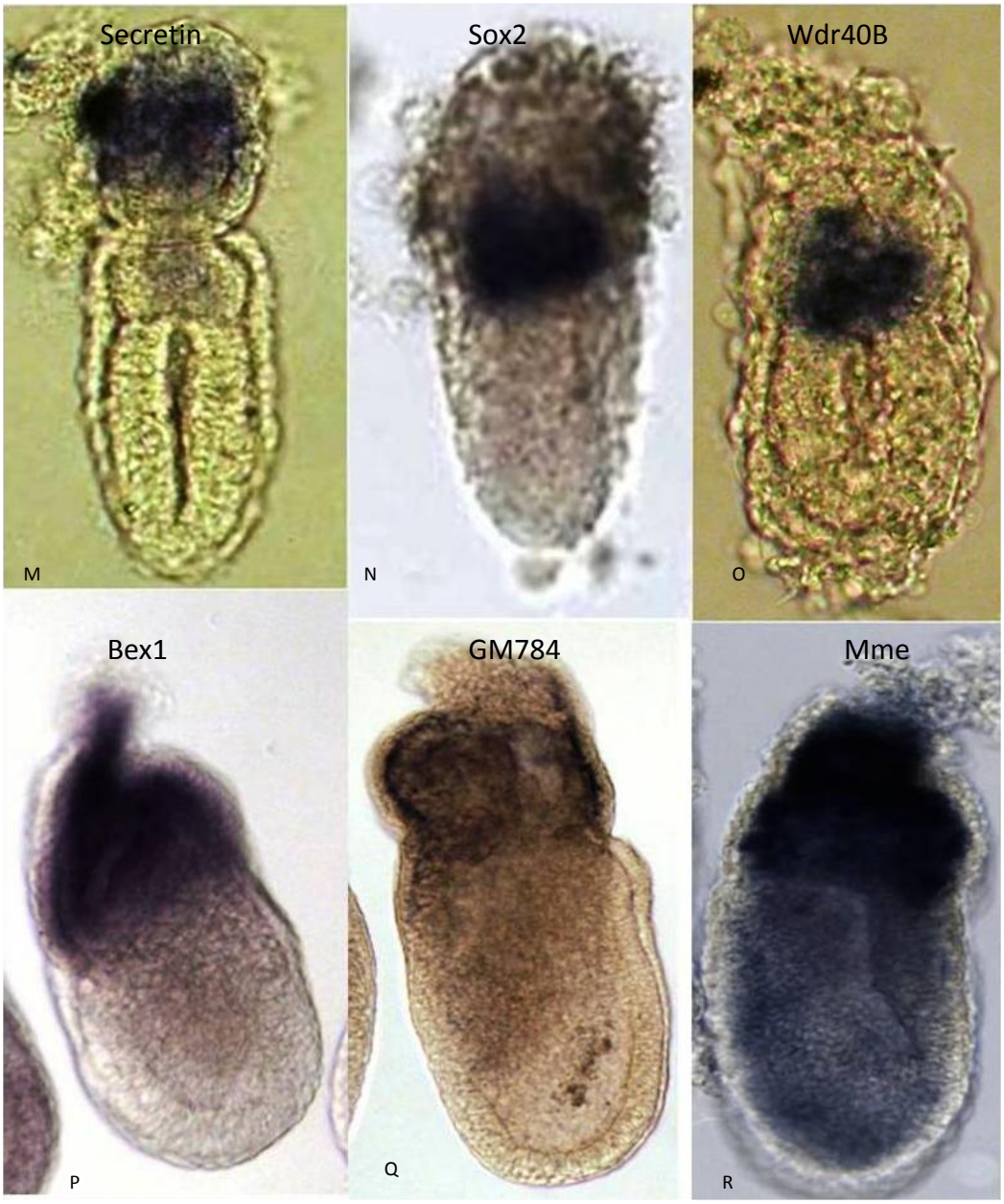


Figure 31: *In vivo* expression of other targets examined by Martyn Donnison. M) *Secretin* was expressed in the EPC, N) *Sox2* was expressed in the ExE, O) *Wdr40B* was expressed in the ExE, P) *Bex1* was expressed in the ExE, Q) *GM784* has mosaic expression in the ExE, and R) *Mme* was expressed in the ExE. M-O are E6.5, P-R are E7.5.

4.2.3 Summary

4.2.3.1 Changes in gene expression upon *Elf5* Knockdown

Table 13: Summary of Elf5 targets. This table shows where the targets are expressed and whether they are up or down regulated upon the loss of *Elf5*.

Down Regulated	*Cyr61*	*Sox2*	*Wdr40B*	*Mme*	*Calca*	*Zic3*
Expressed	ExE	ExE	ExE	ExE	Node	Epiblast
Down Regulated	*Elavl2*	*Spi8*	*Hmga2*	*GM784*	*Bex1*	*1700112c13*
Expressed	ExE	ExE	?	ExE	ExE	Chorion?

Up Regulated	*Ly6a*	*Secretin*	*Keratin7*	*Hopx*
Expressed	ExE	EPC	EPC	Giant cells

The majority of the genes that were down regulated upon *Elf5* knockdown were expressed in the ExE, see Figure 29, Figure 30 and Figure 31. This suggested that Elf5 normally acts to maintain or up-regulate the expression of these genes. This provided evidence for the theory that *Elf5* acts to maintain the ExE. Most of these genes were also down regulated when differentiation of the TS cells was induced. This also provided support for the theory that Elf5 maintains TS cells in an undifferentiated /proliferative state and prevents differentiation into the EPC see Figure 5 (see also corroborative evidence in chapter 6). *Calca* was expressed in the node. *Calca* mRNA levels in TS cells (determined by real time PCR) were quite low, around 0.01-0.02 as compared to *Elf5* expressed at 0.5 (relative to the geomean). This could mean that *Calca* levels were too low in the ExE to be picked up by whole mount *in situ* hybridisation. The development of colour could not be left for any longer as *Calca* was also expressed in the node and this staining would spread over the entire embryo.

The genes that were up regulated upon the knockdown of *Elf5* were expressed in the EPC or giant cells. The exception was *Ly6a*, but I am unsure whether this gene was a target of Elf5 as a large si negative effect was seen in the 24 hr knockdown and then at 48 hr one siRNA caused up-regulation of *Ly6a* and the other caused down regulation. *Secretin, Keratin7* and *Hopx* were expressed in

the EPC or giant cells and were usually down regulated or their expression was restricted by *Elf5*. This also provided evidence for our initial theory that Elf5 acts to maintain the ExE by preventing differentiation into EPC or giant cells. Elf5 prevents the up-regulation of these genes and therefore is helping prevent differentiation into this subtype. *Keratin7* and *Secretin* were up-regulated when differentiation was induced. *Hopx* was not up-regulated when differentiation was induced according to the results attained here.

5 Chapter Five: Over-expression of Elf5 by the tamoxifen inducible system

5.1 Overview

Although the *Elf5* knockdown and whole mount *in situ* hybridisation are all strong evidence for the target genes to be valid, it would be interesting to see if we could get the opposite effect upon gain of function. Therefore we wanted to overexpress Elf5 in mouse trophoblast stem cells. To see a change in gene regulation upon the over expression of Elf5, an inducible system can be used. The classical overexpression system is the Tet system. The tet system relies on two components, a tetracycline controlled transactivator and a Tet responsive promoter that controls the expression of downstream cDNA (the gene of interest). This system is tightly controlled by the presence or absence of tetracycline or a derivative like doxycycline (Jaisser, 2000). This system requires two stable transfections, the first with the regulator plasmid to generate stable cell lines. And then with the response plasmid containing gene of interest under control of the responsive promoter. This system has caused great difficulties in our lab (see Deane, (2007)) so other possible methods for over expression were explored. The tamoxifen system can be used to over express proteins of interest. The tamoxifen system contains a mutated estrogen ligand binding domain (ER-T2) that binds synthetic antagonists such as tamoxifen but not circulating estrogens (Jaisser, 2000). In this system, 4-Hydroxytamoxifen (OHT) a metabolite of tamoxifen is used instead of tamoxifen as it has a higher affinity for estrogen receptors. This domain is fused to the protein of interest and is placed down-stream of a strong cytomegalovirus IE promoter. For example, Niwa et al,. (2005) used a 4-hydroxytamoxifen-inducible Cdx2 which was fused to the ligand binding domain of the mutant mouse estrogen receptor. When expressed in ES cells they could be induced to differentiate into trophectoderm in the presence of Fgf4.

To study the over expression of Elf5, TS cells can be transfected with a plasmid containing the *Elf5* gene fused to an ERT2 receptor. Upon addition of 4-hydroxytamoxifen (OHT), ERT2 binds to this ligand and undergoes a conformational change resulting in it no longer binding to a heat shock protein in

the cytoplasm. The heat shock protein would have kept the fusion protein in the cytoplasm and therefore prevented DNA binding (Danielian et al., 1993). Upon the addition of OHT Elf5-ERT2 thus is free to translocate to the nucleus and fulfil its transcriptional functions. The plasmid also contains the gene for zeocin resistance downstream of the fusion protein. Thus TS cells stably transfected with this vector, can be selected by zeocin resistance. These cells have the plasmid inserted in their genome. Zeocin is a member of the bleomycin family of antibiotics that were isolated from *Streptomyces*. Zeocin is active against bacteria, fungi, plants and mammalian cell lines. Zeocin causes cell death by binding to DNA and cleaving it. Zeocin resistance is encoded by the *Sh ble* gene, which encodes a protein which binds to Zeocin to prevents DNA cleavage activity (Invitrogen, 2002). Therefore transfected cells can be selected for with Zeocin by the expression of the *Sh ble* gene on the construct of interest.

Once stable cell lines are generated, Elf5 can be activated by addition of 4-hydroxytamoxifen (OHT). Cells can then be harvested for target gene analysis by real time PCR.

5.2 Transfection of Mouse Trophoblast Stem Cells

5.2.1 Strategy One:

5.2.1.1 Initial transfection of mTS cells with Elf5 mo ER pyCAGizeo

The initial construction of this plasmid and the stable transfection of the mouse trophoblast stem (TS) cells were carried out by Craig Smith. Craig encountered immense difficulties trying to get this plasmid constructed, as there are inherent problems with this plasmid. The plasmid used initially to transfect the TS cells for the over expression of Elf5 is shown below in Figure 32.

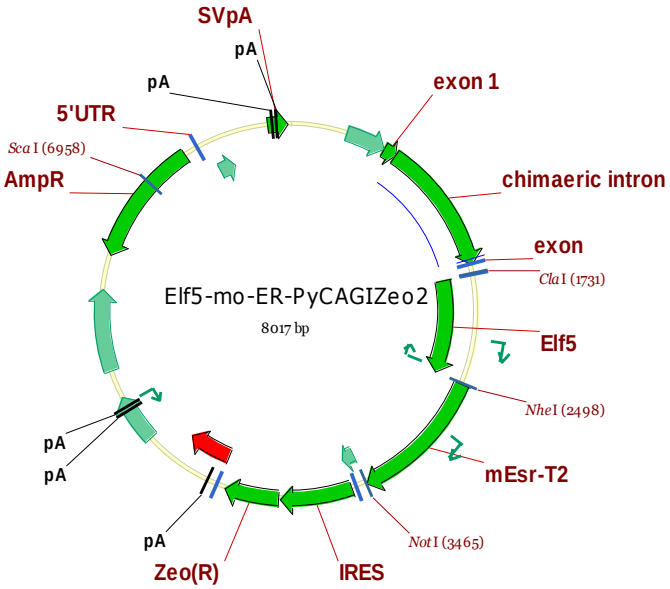
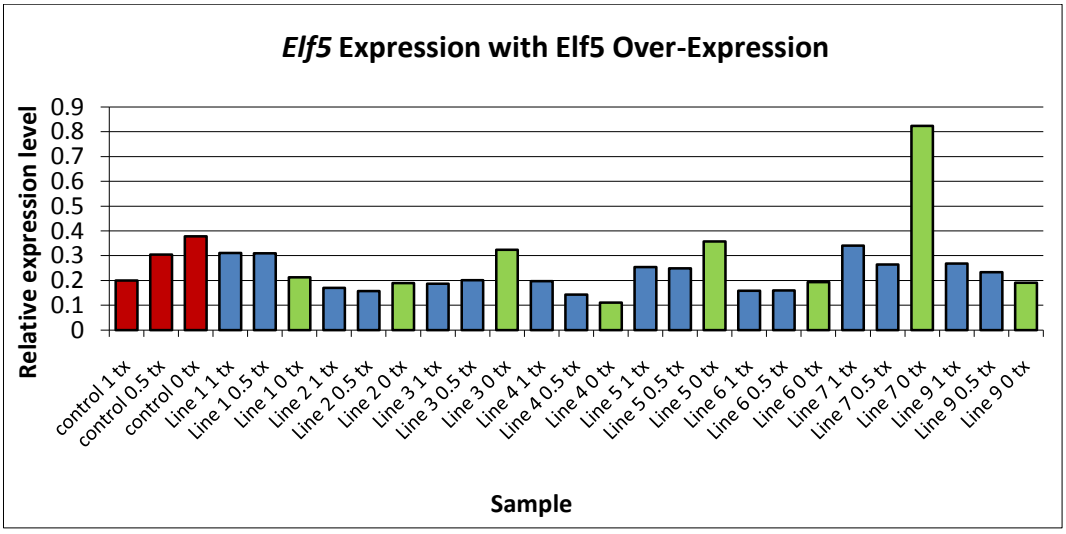
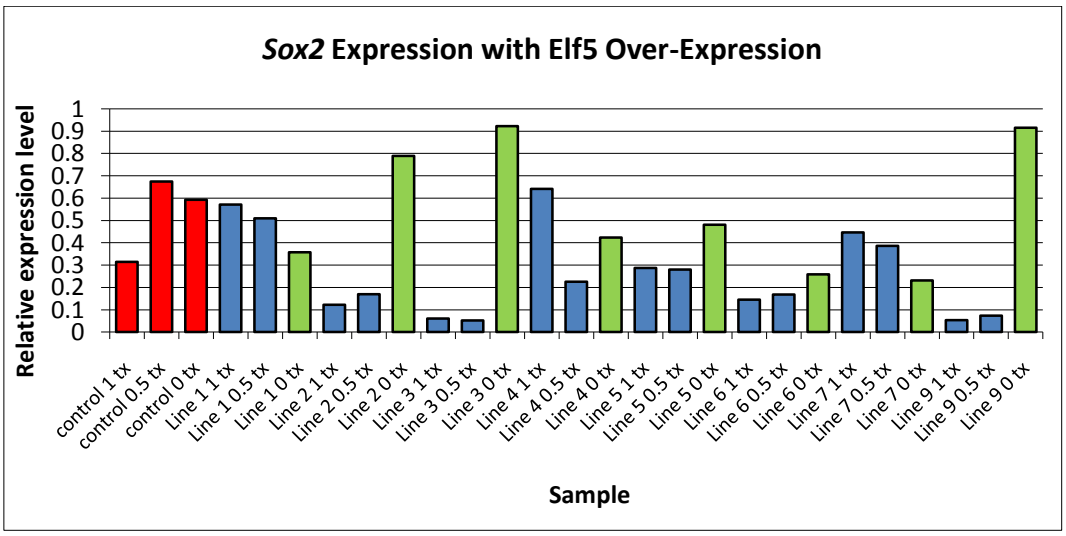
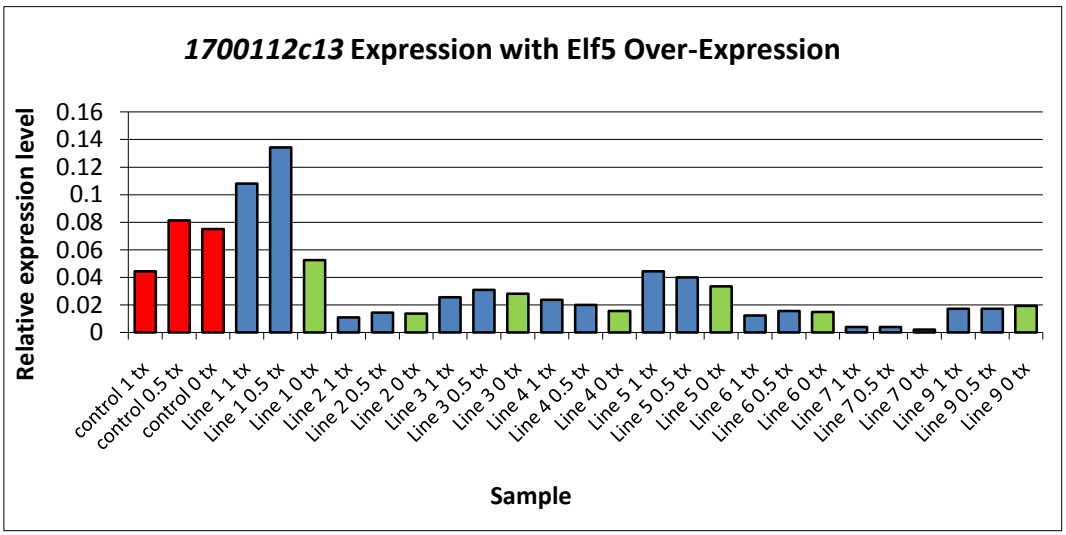


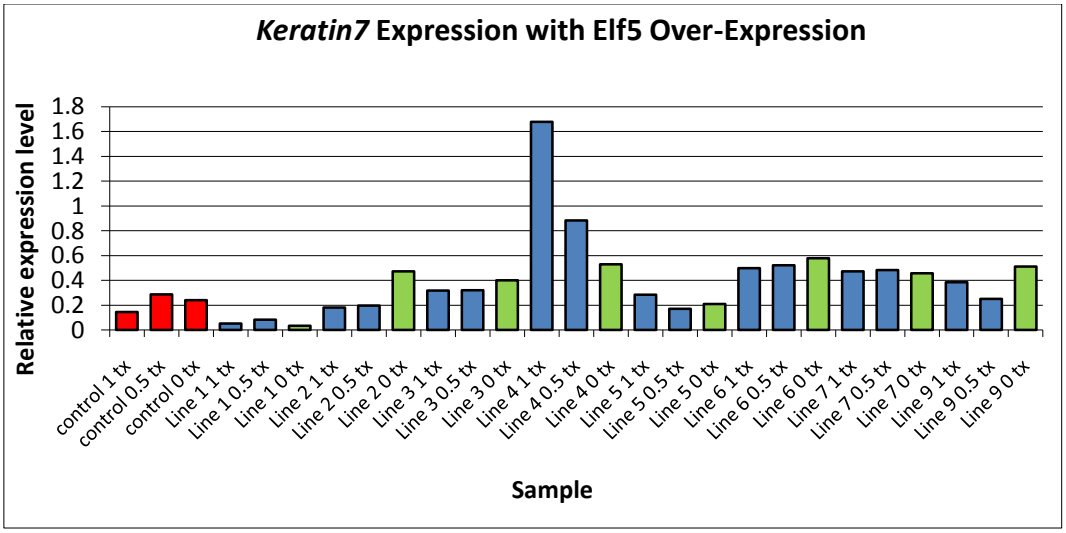
Figure 32: Elf5 mo ER PyCAGiZeo plasmid which was used as a start point for the 4-hydroxytamoxifen inducible system, Elf5 is fused to the Esr-T2 receptor.

Craig stably transfected TS cells with this plasmid to over express the Elf5 protein and adhered to the protocol described in 2.1.4. Craig performed the RNA isolations and cDNA synthesis and gave me the cDNA to perform real time PCR, method described in section 2.3.1.5. The expression of some of the Elf5 target genes were analysed to see if there was a specific up or down regulation of the target genes as expected. The expression of *Elf5*, *Sox2, Ly6a, 1700112c13* and *Keratin7* was investigated, Figure 33.




Sox2 Expression with Elf5 Over-Expression
Relative expression level
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
control 1 tx
control 0.5 tx
control 0 tx
Line 1 1 tx
Line 1 0.5 tx
Line 1 0 tx
Line 2 1 tx
Line 2 0.5 tx
Line 2 0 tx
Line 3 1 tx
Line 3 0.5 tx
Line 3 0 tx
Line 4 1 tx
Line 4 0.5 tx
Line 4 0 tx
Line 5 1 tx
Line 5 0.5 tx
Line 5 0 tx
Line 6 1 tx
Line 6 0.5 tx
Line 6 0 tx
Line 7 1 tx
Line 7 0.5 tx
Line 7 0 tx
Line 9 1 tx
Line 9 0.5 tx
Line 9 0 tx
Sample


1700112c13 Expression with Elf5 Over-Expression
Relative expression level
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
control 1 tx
control 0.5 tx
control 0 tx
Line 1 1 tx
Line 1 0.5 tx
Line 1 0 tx
Line 2 1 tx
Line 2 0.5 tx
Line 2 0 tx
Line 3 1 tx
Line 3 0.5 tx
Line 3 0 tx
Line 4 1 tx
Line 4 0.5 tx
Line 4 0 tx
Line 5 1 tx
Line 5 0.5 tx
Line 5 0 tx
Line 6 1 tx
Line 6 0.5 tx
Line 6 0 tx
Line 7 1 tx
Line 7 0.5 tx
Line 7 0 tx
Line 9 1 tx
Line 9 0.5 tx
Line 9 0 tx
Sample


Keratin7 Expression with Elf5 Over-Expression
Relative expression level
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control 1 tx
control 0.5 tx
control 0 tx
Line 1 1 tx
Line 1 0.5 tx
Line 1 0 tx
Line 2 1 tx
Line 2 0.5 tx
Line 2 0 tx
Line 3 1 tx
Line 3 0.5 tx
Line 3 0 tx
Line 4 1 tx
Line 4 0.5 tx
Line 4 0 tx
Line 5 1 tx
Line 5 0.5 tx
Line 5 0 tx
Line 6 1 tx
Line 6 0.5 tx
Line 6 0 tx
Line 7 1 tx
Line 7 0.5 tx
Line 7 0 tx
Line 9 1 tx
Line 9 0.5 tx
Line 9 0 tx
Sample

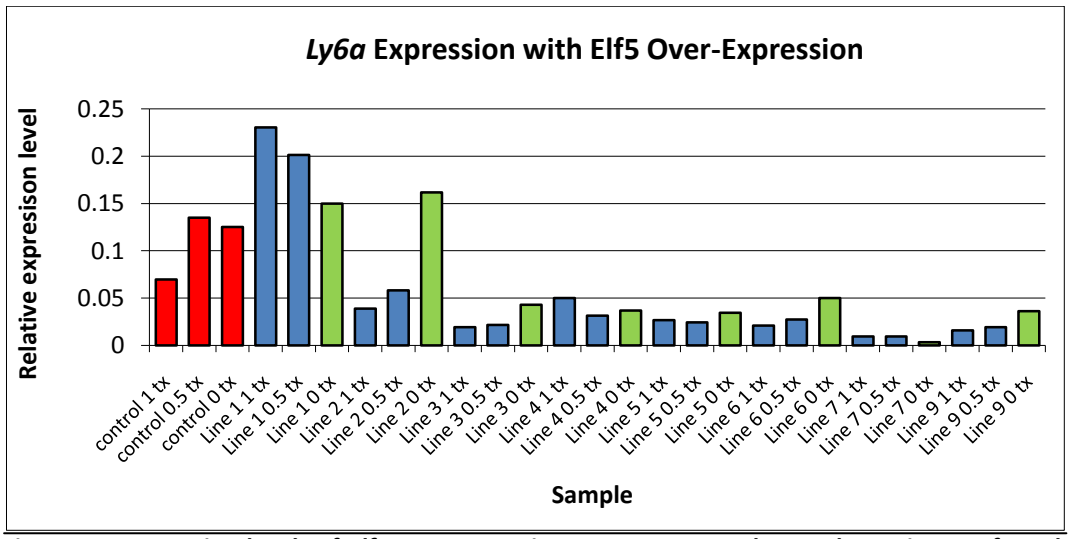


Figure 33: Expression levels of *Elf5, Sox2, Keratin7, 1700112c13* and *Ly6a* shown in transfected mouse trophoblast stem cells. Expression is shown relative to the geometric mean of three house-keeping genes. N=1. Two concentrations of 4-hydroxytamoxifen were used, 0.5 µg/ml and 1 µg/ml. The red bars are the control and green bars are O OHT.

No trend could be identified as an up or down regulation of the target genes. There were no cell lines that showed a higher level of *Elf5* mRNA in comparison to the control. The addition of 4-hydroxytamoxifen (OHT) seemed to have an effect on the house keeping genes *gapdh* and *βactin*. The addition of OHT also seemed to up regulate *Eomes* in the control lines (graph not shown), which is a marker of TS cells. The addition of OHT alone also caused a change in target gene expression in the control cell lines. Careful consideration will need to be taken when repeating this transfection in terms of controls. This experiment did not seem to work as *Sox2* was down regulated upon the addition of OHT in most cell lines. *Sox2* was assumed to be up-regulated when Elf5 is over expressed as it is down regulated when *Elf5* is knocked down. Different lines showed different trends in terms of the changes in gene regulation upon the addition of OHT. This experiment was abandoned.

5.2.2 Strategy Two: Addition of VP16

The lack of effect upon the over expression of this fusion protein (ERT2-Elf5) suggested a novel approach was required to make a stronger activator out of Elf5 by fusing it with VP16. The VP16 domain is a potent transcriptional activation domain (Ghosh et al., 1996). This system has been used by others as a fusion protein with GAL4-ER to allow the activation of transcription from the GAL

promoter in the presence of estradiol (Quintero et al., 2007). In the Tet-advanced system three minimal 'F' type transcriptional activation domains from the VP16 protein are used in place of the full domain. This provides the same activation as the full product but it reduces cross reactivity with other transcriptional machinery and cytotoxicity to the cells (Baron et al., 1997). The VP16 transactivation domain of the herpes simplex virus was added to our Elf5-ER plasmid to ensure robust transcription (Nishioka et al., 2009).

This plasmid was prepared by subcloning the VP16 fragment from the pTet-advanced plasmid; refer to section 2.3.2 of methods. The new plasmid to be created is shown in Figure 34 below. The VP16 cassette was inserted in between the Elf5 and Esr-T2 proteins as described below.

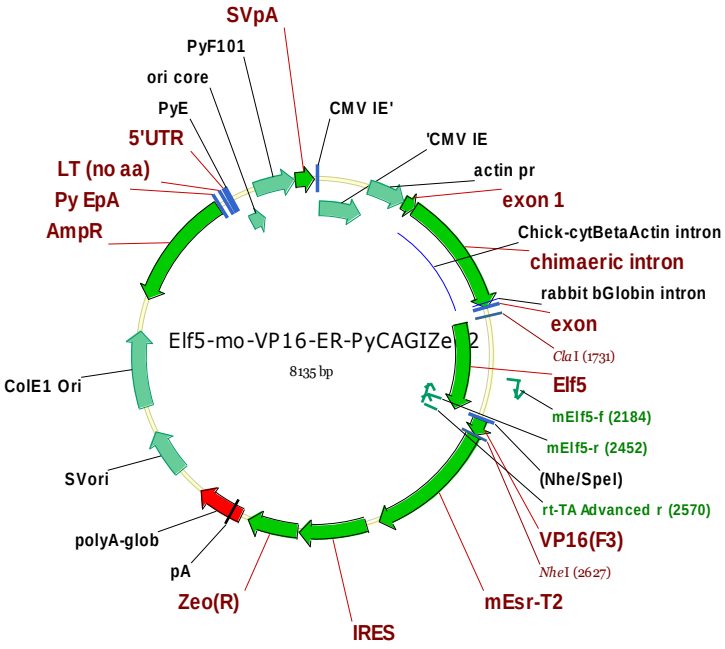


Figure 34: The Elf5 mo VP16 ER pyCAGiZeo plasmid. The VP16 cassette is to be inserted between the Elf5 and EsR-T2 gene in the plasmid.

5.2.2.1 Restriction of Elf5 mo ER pyCAGiZeo

The Elf5 mo ER pyCAGiZeo plasmid was cut at the Nhe I site which is between the *Elf5* gene and the *EsrT2* gene to allow the incorporation of the VP16 cassette into the Elf5 mo ER pyCAGiZeo the vector (refer to Figure 32). This was the site that the VP16 was inserted into. The plasmid was cut in a 20 µl reaction as in 2.3.2.1 at 37 °C for 2 hr. The plasmid was then dephosphorylated (refer to 2.3.2.2) to

prevent the ends from re-joining during ligation and then run on a 1 % gel. For an unknown reason there was an unexpected faint 500 bp band in the digestion product. Due to this the Elf5 mo ER pyCAGiZeo plasmid was digested with Cla I and Not I enzymes to ensure the plasmid was in fact the correct one. Products of the expected size were obtained.

5.2.2.2 PCR amplification of the VP16 cassette

The VP16 cassette was obtained from the pTet-Advanced plasmid by PCR amplification. Genes of interest can be amplified for cloning by using primers containing a 5' restriction sequence of choice and a 3-4 bp clamp before the restriction site. The clamp is inserted as enzymes do not cut optimally at the end of fragments. The primers designed have restriction sites in them so that the restriction sites can be used for insertion into the Elf5 mo ER pyCAGiZeo plasmid. The sequence of the primers used to amplify the VP16 cassette were; VP16-Spe-s ctctACTAGTccggccgacgcccttgacgatt and VP16-Nhe-a ctctGCTAGC accggggagcatgtcaaggtcaa. The ctct at the beginning of each primer is a clamp. The VP16 cassette was PCR amplified by using the conditions stated in 2.3.2.3. The concentration of the pTet-Advanced plasmid was 2.4 µg/µl so the plasmid was diluted 1:3000 to obtain a suitable starting amount for PCR (to about 1 ng/µl).

5.2.2.3 Gel purification of the VP16 cassette

The PCR amplified VP16 cassette was run on a 1.2 % gel and a strong band was obtained at the expected size of 130 bp. The VP16 band was cut out of the gel, weighed and purified from the gel using a wizard column as in 2.3.2.4.1.

5.2.2.4 Restriction of the VP16 cassette

To remove the clamp and to get the cassette ready for ligation into Elf5 mo ER pyCAGiZeo, the purified DNA was cut with Spe1 and Nhe1.

5.2.2.5 Gene Clean of the VP16 cassette

The restricted VP16 cassette was run on a 1.5 % gel and then purified using GENECLEAN. The protocol outlined in 2.3.2.4.2 was followed. A few microliters of the product were run on a gel after purification with GENECLEAN. No product was visible at 130 bp. After reading through the gene clean product information it was discovered that it was not optimal for the purification of products less than 200 bp long.

5.2.2.6 Repeat of VP16 amplification and purification

The PCR amplification of the VP16 cassette was repeated, it was purified on a Wizard column and then restriction digested. The product was then run on a gel. No product at 130 bp was obtained. According to the product information provided with the Wizard columns, a yield of about 80 % is obtained when purifying DNA smaller than 200 bp. But there was obviously a large amount of DNA lost during gel purification so the mermaid kit was only used at both steps (see section 2.3.2.4.3). When the mermaid kit was used instead a faint band was seen at 130 bp. As the yield was low, seven 50 µl PCR reactions were carried out to amplify the VP16 cassette. It was purified using the mermaid kit, the product was restriction digested, and a few microlitres were run on a gel to determine if there was a product. There was a product so it was put through the mermaid column again and eluted in 20 µl and then the product was run on a gel. 1 µl of the VP16 cassette was run on a gel to estimate the concentration against a lambda standard. The concentration of the DNA was estimated to be 4.69 ng/µl, see Figure 35.

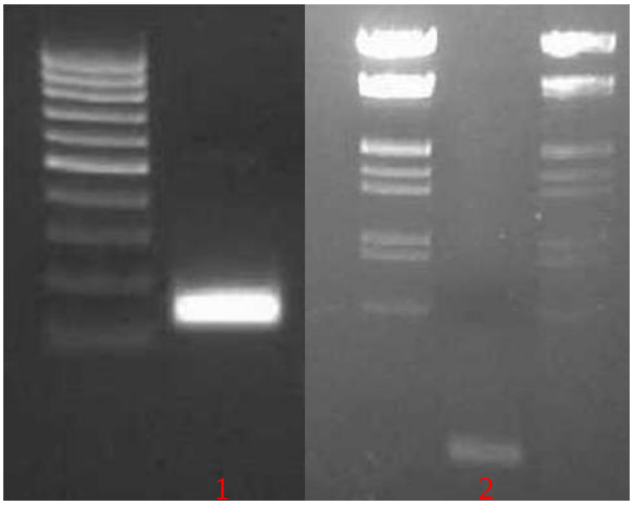


Figure 35: 1= strong VP16 band at 130 bp before gel purification, this was one 50 µl reaction. 2 = 1 µl of VP16 DNA at 130 bp after pooling of 7 PCR reactions, purification, digestion and re-purification. The DNA was run against a lambda standard (both ladders) to give the estimated DNA concentration of 4.69 ng/µl.

5.2.2.7 Ligation of the VP16 cassette into Elf5 mo ER pyCAGiZeo

When performing a ligation equivalent molar ratios of the insert to the plasmid are required. The VP16 concentration was 4.6 ng/µl and the vector concentration was 88.7 ng/µl. The VP16 was 130 bp long while the vector was 8017 bp long which is 1/61. So 2 µl of the plasmid and 1 µl of the VP16 DNA was combined with 3 µl of the Takara mighty mix (see protocol 2.3.2.5). As a negative control the plasmid alone was added to the Takara mighty mix to observe how often the plasmid re-ligates to itself.

5.2.2.8 Transformation into DH5α max efficiency cells

The ligation product was subsequently transformed into DH5α max efficiency competent cells, for a detailed protocol see section 2.3.2.6, and left overnight to grow on LB/Amp plates. No colonies were produced from either the negative control or the sample itself. As no colonies were obtained, the ligation and transformation were repeated again, this time the uncut plasmid was also included as a positive control. No colonies were produced from the positive control or the ligation.

5.2.2.8.1 Repeat of transformation

As no colonies were obtained a new batch of competent cells were thawed and aliquoted out. The cells had been frozen incorrectly by immersion in liquid nitrogen last time so the cells may have died causing this problem. The cells were defrosted on ice, aliquoted out into 25 µl aliquots on ice and then re-frozen in a slurry of dry ice and isopropanol. The transformation was then repeated with these fresh cells, no colonies grew again. Only 2 colonies were obtained from the positive control uncut plasmid. During the same transformation I also transformed 1 pg/µl of Puc19 DNA which is provided with the competent cells. Over 200 colonies were obtained from the Puc19 transformed colonies. This demonstrates that there was nothing wrong with the competent cells.

5.2.2.9 Restriction digest of Elf5 mo ER pyCAGiZeo

As the cells were not the problem, either the ampicillin resistance on the plasmid was not there or not being transcribed or there was something wrong with the origin of replication. So the Elf5 mo ER pyCAGiZeo plasmid was digested with restriction enzymes in varying places in the plasmid, including in the Amp resistance gene to see if products of the correct size were produced. The enzymes used were ApaI, BamHI, EcoRI, SacIII, XhoI to check the plasmid itself and then ScaI and BamHI in the same reaction to check the Amp resistance. All products were the correct size.

5.2.2.10 Miniprep of Elf5moERpyCAGiZeo

As the plasmid itself was fine in terms of sequence, there was something wrong with the growth of this plasmid. So a new preparation of the plasmid was sought out. Due to this some colonies from the glycerol stock of the plasmid were plated out and a miniprep (see protocol 2.3.2.7) of a few colonies was carried out. A miniprep of the few colonies that grew on the positive control plates from an earlier transformation was also prepared. This DNA was digested with Nco I and all minipreps gave fragments of the expected sizes.

5.2.2.11 Maxi prep of Elf5 mo ER pyCAGiZeo

A maxi prep (see protocol 2.3.2.9) from one of these colonies was performed to give a DNA concentration of 2770 ng/µl in 100 µl T.1E. The 260/280 ratio was 1.87 and the 230/280 ratio was 2.23 so the DNA was pure. This DNA was the new stock of the plasmid for further work.

5.2.2.12 Re-digestion of Elf5 mo ER pyCAGiZeo

This newly purified plasmid was digested again with Nhe I, to ensure the plasmid was cut with Nhe I, 1 µl was cut with Cla I and a fragment at 767 bp was produced as expected. The rest of the digested plasmid DNA was dephosphorylated. The ligation and transformation was repeated along with the controls. About 50 colonies grew on one plate and about 100 colonies on the other. 1pg of control uncut plasmid gave 100 colonies as well, as a positive control.

5.2.2.13 Miniprep of Elf5 mo VP16 ER pyCAGiZeo colonies

The miniprep was repeated with 24 colonies and the DNA was digested with Nhe I and Cla l. If the plasmid contained the insert of interest a DNA band at 896 bp would be expected. If there was not an insert or if it was in the reverse orientation a fragment at 774 bp would be expected. The digested DNA was run on a high percentage gel to ensure a good separation between the two DNA bands. All colonies had a DNA band a 774 bp, see Figure 36. Another 48 minipreps were carried out but no bands of the correct size were obtained. Either the plasmid was re-ligating to itself (i.e. the CIPing didn't work) or there was something wrong with the VP16 cassette. As a different calf alkaline phosphatase was used, the plasmid was dephosphorylated again with a fresh batch.

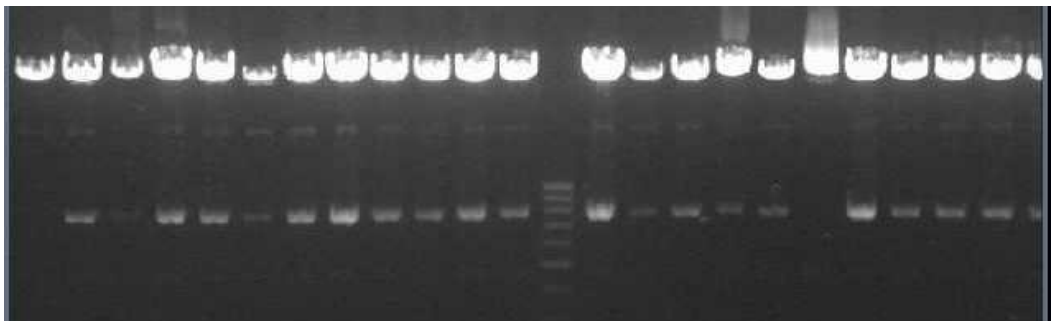


Figure 36: Gel showing the results from a mini prep of clone DNA that was then restriction digested to see if the correct insert was in the clone. All clones either had no insert or they were in the wrong orientation, all products were 774 bp. If they had the correct insert the product size would have been 896 bp.

5.2.2.14 Ligation of VP16 into pGEM-Teasy

As the method above was clearly not working the VP16 cassette was ligated into the pGEM-Teasy plasmid (protocol see 2.3.3.5) which allowed blue/white selection of colonies containing an insert. As a modification to the protocol, after the PCR amplification of the VP16, it was not cut with SpeI or NheI to remove the clamp. This time a lot of colonies were produced, the vast proportion were blue (i.e. they do not have an insert) but there were also plenty of white colonies.

5.2.2.15 Miniprep and restriction digestion

A miniprep of 24 white colonies was completed and they were digested with Eco RV to distinguish if there was an insert. There was no band at the expected size of 130 bp. David Pearton suggested to carry out a PCR to see if the VP16 insert was in the plasmid, all PCR reactions were positive for the VP16 cassette. This suggested that there was not enough DNA from the minipreps to see the small 130 bp DNA fragment on a gel.

5.2.2.16 Midi prep of colonies 1 and 2

To get a higher concentration of plasmid DNA a midi prep was performed and the DNA was digested with Nae I and Sac I. If the insert was present a DNA fragment at 547 bp would be produced, if not a fragment at 417 bp would be present. Both plasmids had a DNA fragment at approximately 550 bp suggesting the VP16 insert was present, refer to Figure 37.

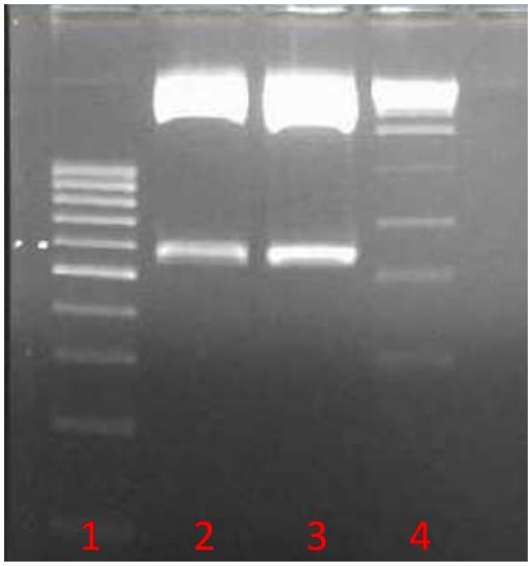


Figure 37: 1= 100 bp ladder, 2 and 3 = digested midi prep DNA showing correct sized DNA bands at 550 bp, 4 = ultra ranger DNA ladder. This means these clones had the correct insert.

Since the VP16 cassette was finally in a plasmid, it was cut out of the p-Gem Teasy plasmid 20 µg of the midi prep DNA was digested in a 50 µl reaction with Spe I and Nhe I to remove to insert.. The VP16 cassette was gel purified and ligated into Elf5 mo ER pyCAGiZeo. The ligation product was transformed into DH5α max competent cells and after the transformation about 100 colonies were obtained.

A miniprep was performed on 24 colonies and then the DNA was digested with Nhe I and Cla I. Only one clone with the correct insert was obtained. A maxi prep of this one clone was prepared and it was digested again to make sure the insert was still there, see Figure 38. A product of the expected size was obtained.

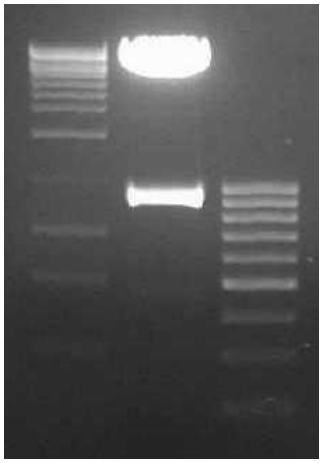


Figure 38: Gel showing maxi prep DNA with the correct size insert at 896 bp

5.2.2.17 Sequencing of Elf5 mo VP16 ER pyCAGiZeo

A few microlitres of the maxi prep DNA was sequenced at the University of Waikato sequencing facility. The primers that were used to sequence verify the insert were mElf5-f TCAAGACTGTCACAGCCGAACAA and VP16-Nhea (reverse primer, sequence given above). When the sequencing results came back it was discovered that there was an insert of 14 bp directly in front of the VP16 sequence.

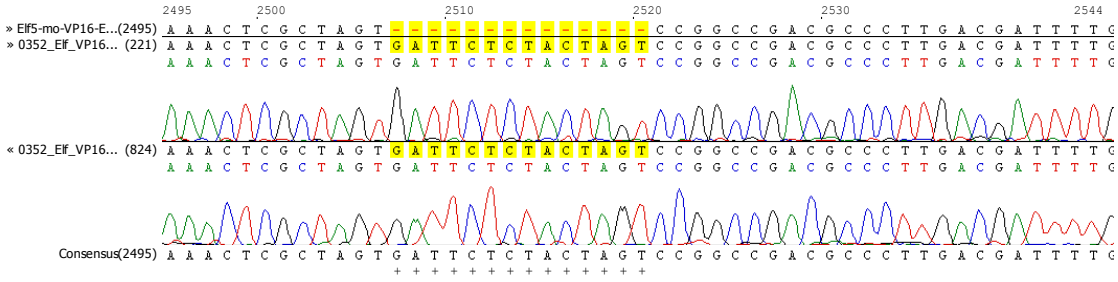


Figure 39: Alignment of Elf5 mo VP16 ER PyCagiZeo (top) against the forward and reverse sequencing reactions. The sequence highlighted in yellow indicates the insert in the maxiprep DNA.

Figure 39 shows the top sequence is the plasmid as it should be (i.e. the correct sequence of the plasmid in vector Nti), the second sequence down is the sequence from the mElf5-f forward primer and the bottom sequence is from the VP16-Nhea reverse primer. Because this insert is visible in both forward and reverse sequence fragments it is real. The chromatograms were also clean so was not a mistake or bad sequencing, especially since both orientations matched.

5.2.2.17.1 The SpeI restriction digest did not work correctly

After searching for this insert in the vector NTi files, this 14 bp insert was found in the pGem TEasy plasmid. Looking back at the p-Gem T Easy plasmid MCS, there was an SpeI site that matches the insert sequence (see Figure 40). So the VP16 insert went into this plasmid in the reverse orientation, was cut out from the plasmid by the SpeI site in p-Gem rather than the SpeI site on the end of the VP16 sequence. The VP16 was then inserted back into Elf5 mo ER pyCAGiZeo in the correct orientation, but accumulating an extra 14 bp to the start of the fragment. This clone could not be used as an insert that is not a multiple of three

(3 bp codes for 1 amino acid) will cause a frame shift mutation and the DNA will no longer code for the correct protein.

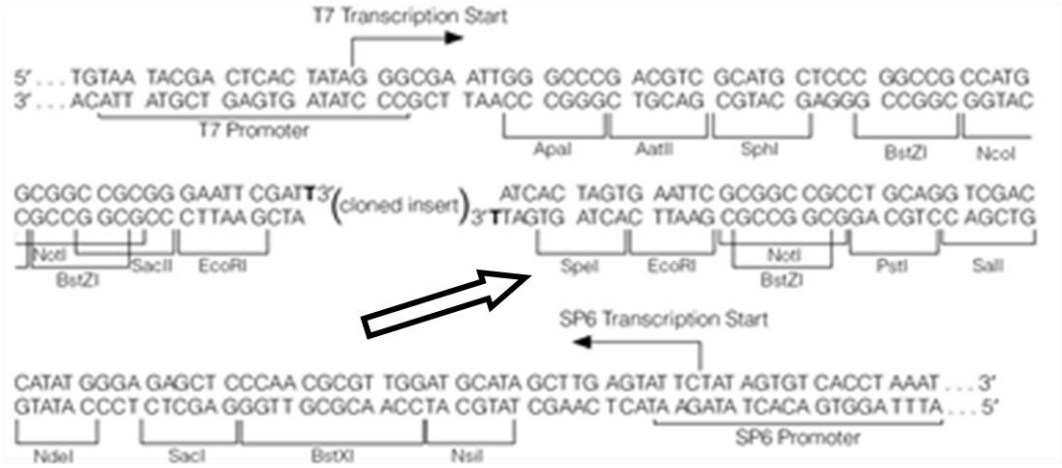


Figure 40: shows how the Spe I digestion went wrong, the restriction digest was not efficient and cut only at the Spe I site on the plasmid and not the one that was also in the fragment, see arrow. This resulted in a 14 bp insert in front of the sequence of interest.

5.2.2.17.2 *Repeat of miniprep*

As the above clone could not be used, 48 more minipreps were prepared of the colonies obtained from the previous transformation. The DNA was digested with ClaI and NheI to check for the insert. 12 clones looked like they may have the insert. The DNA was then digested with SpeI. If the clone had the correct insert it should only cut once with SpeI, if it had the 14 bp insert it would cut twice. The clone that had the 14 bp insert was run alongside the digestion to show what it looked like if it was cut at the wrong SpeI site. The uncut plasmid was also added as another control, see Figure 41 below.

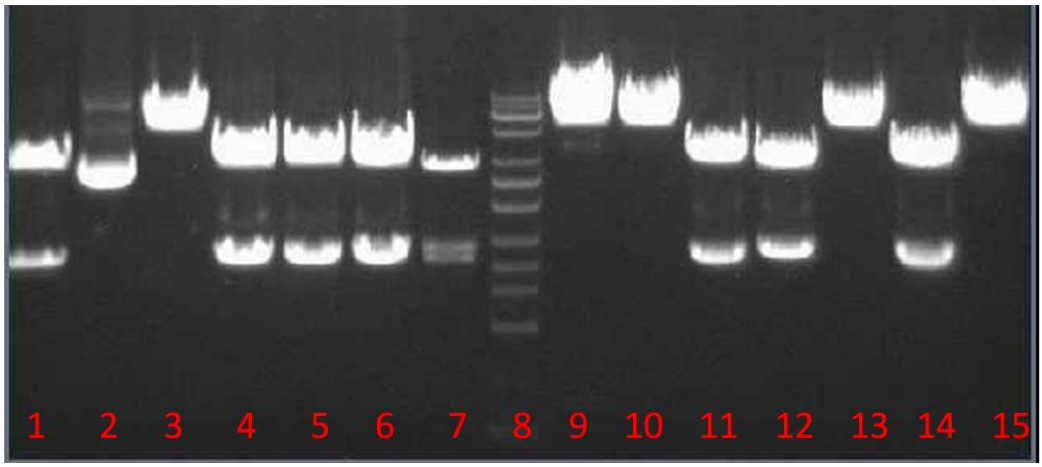


Figure 41: 1 is the clone with the 14 bp insert, 2 = uncut plasmid, 3-15 are different clones, 8 is an ultra ranger ladder. Clones that cut twice also had the 14 bp insert. Clones that cut once were correct.

Clones 4-6, 11-12 and 14 had been cut twice which meant that they too had the 14 bp insert at the start of the VP16 sequence. The initial digestion must have been inefficient for a lot of the clones to have this insert. Clones 10, 13 and 15 had the correct fragment size and were sent for sequencing. Clones 3 and 9 did not have the correct sized fragments when digested with NheI and ClaI they were just added for comparison.

5.2.2.18 Maxiprep and Sequencing

A maxiprep of clones 10, 13 and 15 was prepared and the resulting DNA was sequenced at the University of Waikato sequencing facility. Clone 13 had a perfect sequence in comparison to the Elf5 mo VP16 ER pyCAGiZeo clone chart in an alignment, see Figure 42 below.

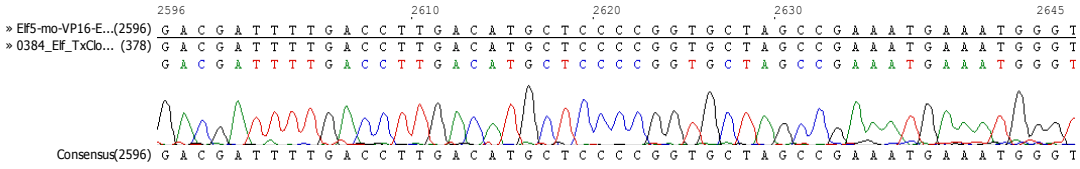


Figure 42: Sequence of the beginning of the VP16 gene aligned with the Elf5 mo VP16 ER pyCAGiZeo file in vector NTi. This prep has the correct sequence and can therefore be used to transfect the cells.

5.2.2.19 Preparation of Glycerol Stock

A glycerol stock was made of clone 13 for later use. A glycerol stock was made with 15 % glycerol and 85 % bacterial suspension, this was flash frozen in liquid nitrogen and then stored at -80 °C.

5.2.3 Stable Transfection of Elf5 mo Vp16 ER pyCAGiZeo

5.2.3.1 Linearization of DNA for transfection

The correct plasmid for transfection of mouse trophoblast stem cells for the over expression of Elf5 was finally produced. Before the TS cells could be transfected with the plasmid DNA, the DNA had to first be linearised for optimal integration into the trophoblast stem cell genome. The plasmid was cut in the Ampicillin resistance gene since that gene was not required once the DNA was in the TS cells. 30 µg of the plasmid DNA was cut with ScaI and then purified through a wizard column (see protocol 2.1.4.1). The DNA concentration was estimated on

the nanodrop so the optimal concentration of DNA could be used to transfect the cells. Surprisingly 10 µg of DNA was lost during the digestion and purification procedure.

5.2.3.2 Transfection of mTS cells number one

The TS cells were transfected with 4 µg of the Elf5 mo Vp16 ER pyCAGiZeo plasmid according to the protocol outlined in sections 2.1.4.2-2.1.4.4. Five transfections were carried out with one untransfected control line. The control line was important to ensure all of the un-transfected cells and therefore susceptible to zeocin die off before resistant colonies can be picked. Zeocin selection began 24 hr after the TS cells had been transfected, refer to section 2.1.4.5.

5.2.3.2.1 Trouble shooting

A few days after the beginning to the zeocin selection the TS cells became completely confluent and some differentiation was beginning to appear. The control cell line was not beginning to die off as it should as these cells do not have any resistance to zeocin. According to the protocol outlined in Himeno et al., (2008) it should take seven to fourteen days for stable transfected colonies to appear when treated with 200 µg/ml zeocin. Upon reading the Zeocin user manual it was discovered that zeocin can take up to 6 weeks to generate resistant cell colonies. Zeocin does not cause cells to round up and detach from the plate like traditional selection antibiotics. This makes it difficult to determine which cells are in fact dead. According to the Zeocin manual the following morphological changes can be observed upon Zeocin treatment; increase in size, abnormal cell shape and the appearance of long appendages, presence of large empty vesicles in the cytoplasm and the breakdown of the plasma and nuclear membrane. Ultimately the cells completely break down and only cellular debris will remain. As the cells were extremely confluent the zeocin concentration was increased to 500 µg/ml one week after the beginning of the transfection. Once there was a large amount of cell death the zeocin concentration was decreased back to 200 µg/ml. After three weeks of selection resistant colonies were beginning to appear in the plates.

5.2.3.3 Kill Curve

As the zeocin concentration used did not seem optimal a kill curve was prepared to determine what concentration of zeocin killed off untransfected cells after 1-2 weeks under antibiotic selection. For a 10 cm^2 dish a seeding density of 5 $x10^5$ cells/ml was required. The kill curve was carried out in 6-well dishes to save media as the media needs to be changes every second day. The area of a 6-well dish is 3.7 cm^2 so a seeding density of 1.85 $x10^5$ cells/ml was required to down scale the experiment accordingly. As 200 µg/ml was the recommended concentration of zeocin to use (Himeno et al., 2008), both lower and higher concentrations of this were tested. The TS cells were plated out the day before at the recommended concentration of 1.85 $x10^5$ cells/ml in 5 wells of a 6 well dish. 24 hr later the media was replaced with 70 % conditioned media containing 100 µg/ml to 600 µg/ml zeocin. The media was changed every second day and the cell death was monitored.

Table 14: Optimisation of Zeocin concentration for trophoblast stem cells. Zeocin was a very slow acting antibiotic.

Zeocin Concentration (ug/ml)	Day 2 (% dead compared to untreated)	Day 6 (% dead compared to untreated)	Day 10 (% dead compared to untreated)	Day 14 (% dead compared to untreated)	Day 18 (% dead compared to untreated)
0	Confluent	0	0	0	0
100	Confluent	0	0	0	10
200	Confluent	0	0	5	50
400	Confluent	0	5	20	80
600	Confluent	0	15	60	100

After two days of antibiotic selection all the cells became confluent and the antibiotic selection didn't seem to have much of an effect on the TS cells. No cell death was observed in any of the wells until 10 days of antibiotic selection. A small amount of cell death was becoming visible in the TS cells treated with 400 and 600 µg/ml of zeocin. After 18 days of selection the majority of the cells were dead in both the 400 µg/ml and 600 µg/ml zeocin wells. No effect was seen with 100 µg/ml zeocin and a minimal effect with 200 µg/ml of zeocin (refer to Table 14). The major problem here seems to be the initial seeding density of the TS

cells is too high which leads to the cells becoming confluent only a few days after the beginning of the antibiotic selection. As zeocin is a slow acting antibiotic the cells become too confluent before it has time to take effect. Next time either the cells density will be decreased or a different antibiotic will be used.

5.2.3.4 Picking of stably transfected cell lines

One month after beginning the stable transfection resistant colonies were beginning to appear in the cell culture dishes. The colonies were of various sizes and some were growing faster than others. To establish clonal cell lines the zeocin resistant colonies were picked according to the protocol outlined in section 2.1.4.6. The following day the media was changed and 0.5 ml of 70 % conditioned media was aliquoted into each well of the 24 well plate. In total 58 colonies were picked. Of the 58 colonies that were picked, only four wells had TS cell colonies that had survived the picking procedure. Apparently this is not uncommon. One and a half weeks after picking, one of the wells had very nice colonies growing fast enough to be able to passage into a well of a 6-well dish. Two of the colonies that were picked differentiated after passaging once into a 12 well plate. This left only two cell lines that were well established.

5.2.3.5 Transfection of mTS cells number two

As the original transfection of the mTS cells with the Elf5 mo VP16ERpyCAGiZeo plasmid was very unsuccessful with only two cell lines being established, the transfection was repeated. This time the cells were co-transfected with a linearised plasmid containing the *pac* gene for puromycin resistance on it. The plasmid was provided by Craig Smith and was digested with EcoRI to linearise. Puromycin is an antibiotic produced by *Streptomyces alboniger* that inhibits the growth of prokaryotic and eukaryotic cells by interfering with protein synthesis by inhibiting peptidyl transfer on ribosomes. Resistance to puromycin is provided by the expression of the *pac* (puromycin N-acetyl transferase) gene (Sigma-Aldrich, 2009). If the cells are transfected with equal amounts of Elf5 mo VP16 ER pyCAGiZeo and the puromycin plasmid it is likely that cells will be puromycin resistant but not contain the plasmid of interest. Due to this 1/10 of the puromycin plasmid was used in relation to the Elf5 mo VP16 ER pyCAGiZeo

plasmid so if cells were resistant to puromycin they would be likely also carry the transgene of interest. The Elf5 mo VP16 ER pyCAGiZeo plasmid was 8135 bp and the puro plasmid was 4706 bp. Which was already a 1:1.7 ratio to get equivalent molar ratios. This could then be divided by 10 to give an optimal DNA ratio of 1/17. So to get the correct 1:10 ratio of Elf5 mo VP16 ER pyCAGiZeo to puromycin resistance, $5x10^5$ cell/ml of TS cells should be transfected with 4 µg of Elf5 mo VP16 ER pyCAGiZeo DNA and 0.24 µg of the puromycin plasmid. 24 hr after the trasfection the cells were selected with 1 µg/ml puromycin (optimal concentration was determined by Deane (2007)). Refer to section 2.1.4.5.1 of methods.

5.2.3.5.1 Trouble Shooting

After one week of selection the control cells were only dying off slowly. This was contrary to what was expected. The control cells should have all be dead within one week of starting antibiotic selection. Dying cells should detach from the plate allowing easy identification of resistant colonies. It was concluded that the initial seeding density of the cells was too high which rendered the antibiotic ineffective as it works best on actively dividing cells. This experiment was abandoned.

5.2.3.6 Transfection of mTS cells number three

This time the initial seeding density was reduced by 2.5 x to $2x10^5$ cells/ml as the seeding density of $5x10^5$ cells/ml was far too high even when using puromycin as the selection antibiotic. The transfection was carried out as outlined in 5.2.3.5 and selection with 1 µg/ml puromycin began 24 hr after the beginning of the transfection. As expected the control cells died off within four to seven days.

5.2.3.7 Integration of the plasmid into the genome

The genomic DNA of the stable cell lines must be analysed for integration of the plasmid DNA into the genome. The genomic DNA could be isolated from a small fraction of cells obtained during passaging (about 1/10 was plenty), see methods 2.1.4.7. The integration of the plasmid DNA into the genome could be verified by PCR. Primers were designed to the zeocin resistance segment of the plasmid DNA

as *Elf5* is also expressed endogenously so was not a good candidate for examination by PCR. Two sets of zeocin primers were used, ZeoF GACGACGTGACCCTGTTCATCA ZeoR GTCCTGCTCCTCGGCCACGAA (product size 243 bp) and Zeo F2 CAGGACCAGGTGGTGCCGGACAA and ZeoR2 CTGCTCGCCGATCTCGGTCATG (product size 141 bp). Figure 43 below shows lines one and two from the first transfection were positive for integration into the genome, the second zeocin primer set was used for this PCR.

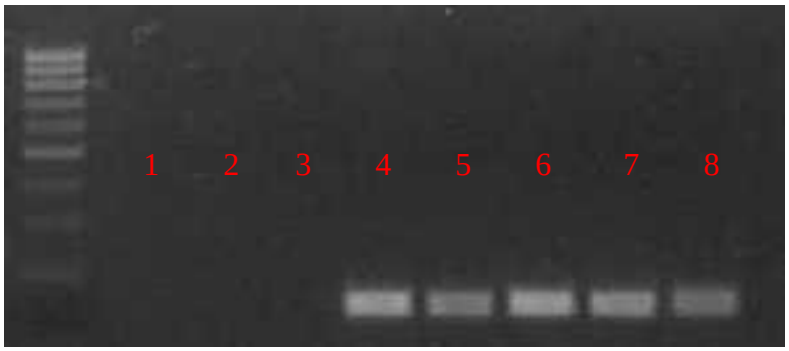


Figure 43: gel of genomic DNA amplified with Zeocin primers. 1= MQ, 2, 3= untransfected TS cells, 4, 5= line 1, 6, 7= line 2, 8= 1:10,000 dilution of the purified Elf5 mo VP16 ER pyCAGizeo plasmid. Both line 1 and 2 contained the plasmid inserted into their genome.

5.2.4 Immunocytochemistry

Immunocytochemistry was carried out on fixed mouse trophoblast stem cells in chamber slides to ensure the cells were expressing the Elf5-VP16-ER protein and that it translocated into the nucleus upon the addition of 4-hydroxytamoxifen. See section 2.1.4.9 for the method of immunocytochemistry. The primary antibody was to the ER-alpha ligand binding domain (c-terminus) of the plasmid (ABBIOTEC, Australia). An Elf5 antibody could not be used as Elf5 is also expressed endogenously.

5.2.4.1 Trial one:

Control mouse trophoblast stem cells and line 2 cells were passaged in 8 well chamber slides and then induced by the addition of 1 µg/ml of 4-hydroxytamoxifen 24 hr after passaging, one well was not induced as a control. 24 hr later the cells were fixed with 4 % PFA at RT for 5 min and stored in the fridge until ready to use. The protocol outlined in 2.1.4.9 was followed. Two

concentrations of the primary antibody were tested- 1:200 and 1:400 as a concentration of 1:200-1:500 was recommended for immunocytochemistry by the suppliers. The secondary antibody was a donkey anti-rabbit antibody conjugated to Alexa 488, the concentration used was 1:1000 as this concentration had been successfully used in flow cytometry (refer to chapter six). Unfortunately no fluorescence was observed upon excitation at 495 nm. The H33342 gave very strong staining when excited with a UV light.

5.2.4.2 Trial two:

As the above experiment did not work this was repeated. One well was used as a no primary antibody control to show the level of non specific binding. As a positive control an antibody to H3 serine 10 phosphorylation was used in another well to ensure the protocol and the secondary antibody was working. A 1:200 dilution of the primary antibody was used as this was shown to be optimal for flow cytometry (see section 6.2.3.1). The ER-alpha primary antibody was used at a concentration of 1:100 and the secondary was used at 1:500. The cells were also fixed in methanol this time. The layout of the experiment is shown in Table 15 below.

Table 15: the layout of the chamber slide used for immunocytochemistry.

	Un-induced	Induced	Induced	Induced
Control	1:100 1°, 1:500 2°	1:100 1°, 1:500 2°	1:500 2°	1:200 H3s10 1°, 1:500 2°
Line 2	1:100 1°, 1:500 2°	1:100 1°, 1:500 2°	1:500 2°	1:200 H3s10 1°, 1:500 2°

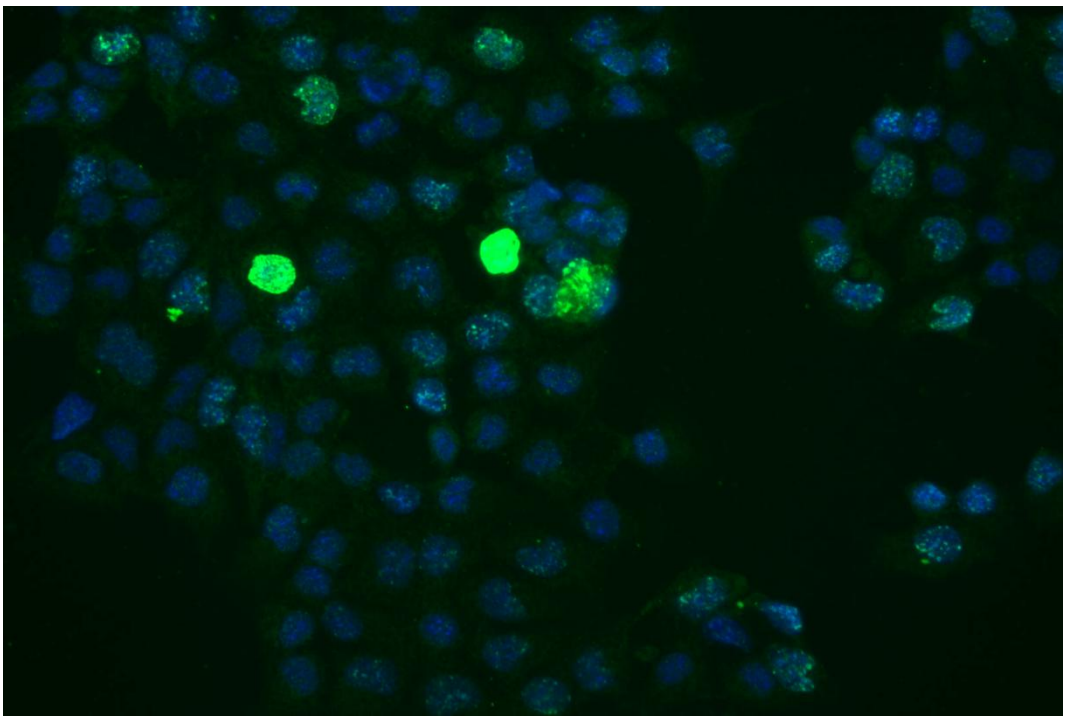


Figure 44: photograph of the H3ser10 antibody on control mouse trophoblast stem cells. Strong green fluorescence was seen in the mitotic cells. The blue fluorescence is the H33342, a nuclear dye. This photograph was taken at 10x magnification.

Strong fluorescence was observed with the histone 3 serine 10 antibody, see Figure 44 above. Only cells in the late G2/M phase of the cell cycle were labelled intensely with this antibody.

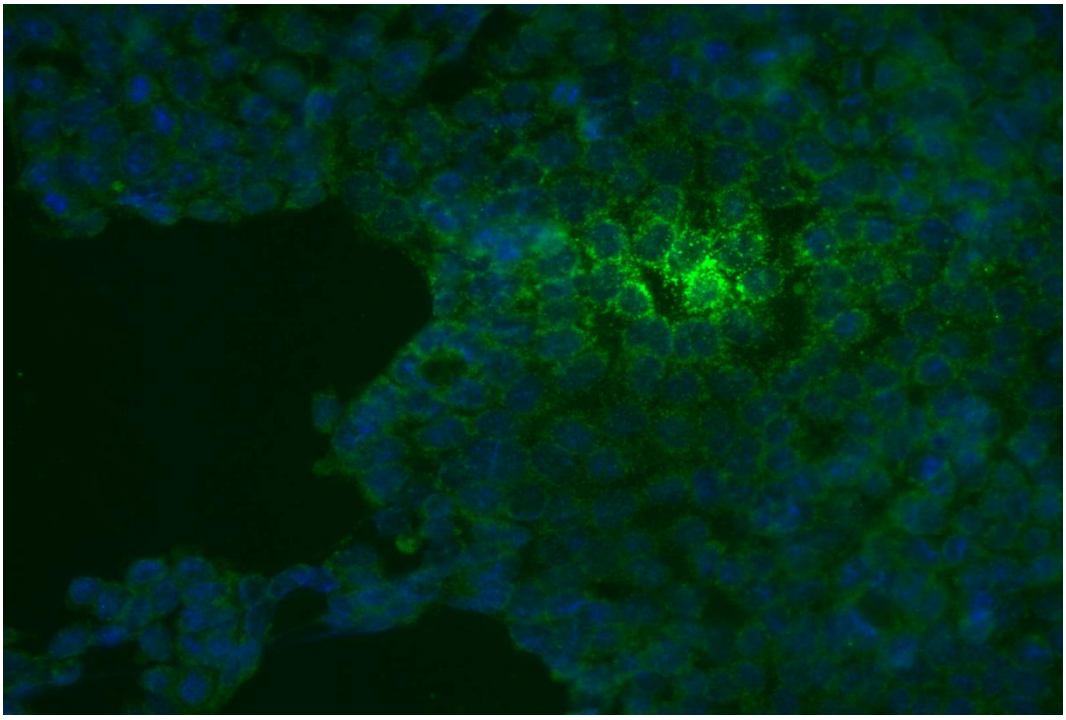


Figure 45: Mouse trophoblast stem cell line 2 un-induced i.e. no tamoxifen has been added. Er alpha antibody binding in the cytoplasm is the green fluorescence. The blue staining is the H33342 in the nucleus. This photograph was taken at a 10x magnification.

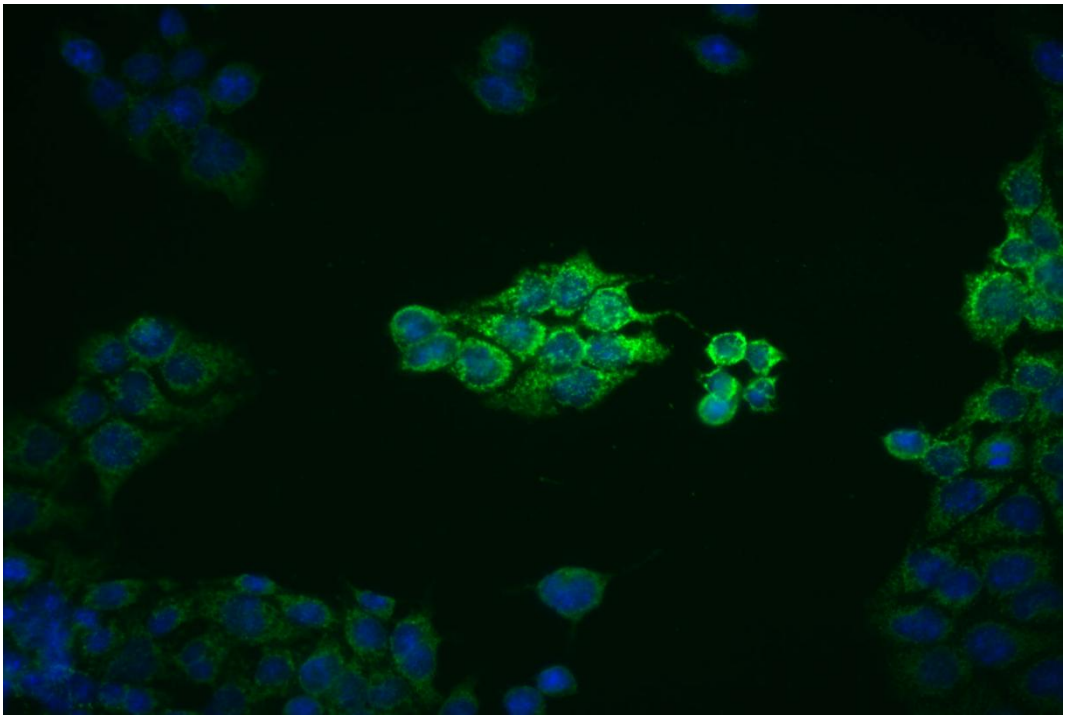


Figure 46: Mouse trophoblast stem cell line 2 with 1 µg/ml tamoxifen. Er alpha antibody binding in the cytoplasm is the green fluorescence. The blue staining is the H33342 in the nucleus. This photograph was taken at a 10x magnification. A lot of the protein generated from the exogenous plasmid still seems to be in the cytoplasm.

The fluorescence observed with the Estrogen receptor antibody was not very strong. The protein was expressed in the cytoplasm as expected in line 2 (see Figure 45), but upon the addition of 4-hydroxytamoxifen there did not seem to be a big shift into the nucleus (see Figure 46). There was some expression in the nucleus but not all of it moves to the nucleus as first expected. Possibly only a fraction of the protein moved to the nucleus upon the addition of OHT. The level of protein expression also was not as high as expected.

5.2.4.3 4-hydroxytamoxifen assay optimisation

Two lines were obtained from the first transfection and they were tested for their induction of Elf5 over-expression in the presence of 4-hydroxytamoxifen (OHT). As it is the protein that is over expressed and not the gene we could not use real time PCR to analyse any changes in *Elf5* upon the addition of OHT. Instead changes in the Elf5 targets had to be analysed. Two different concentrations of OHT were used, 0.5 µg/ml and 1 µg/ml (Niwa et al., 2005) for 24 hr, as well as an untreated control. The OHT was left for 24 hr and then Trizol

was added to each well for RNA isolation (see methods 2.1.4.8). Untransfected cells were also treated with OHT to ensure it was not affecting normal gene expression as OHT can interfere with Errβ expression which is a gene involved in TS cell maintenance (Rielland et al., 2008). The expression of some of the target genes as well as the house keepers were analysed. The expression of the TS stem cell genes *Eomes* and *Cdx2* were not changed upon the addition of OHT and their expression levels were similar for each cell line relative to the control cell line. The expression of *Elf5* was unchanged in line one and 2-3 times higher in line two compared to the control (see Figure 47). There was no change in *Elf5* expression upon the addition of OHT. The *Elf5* primers will pick up both the introduced *Elf5* cDNA and the endogenous cDNA. This indicates that line two has higher expression levels due to expression from the introduced plasmid. Refer to the raw data in the appendices.

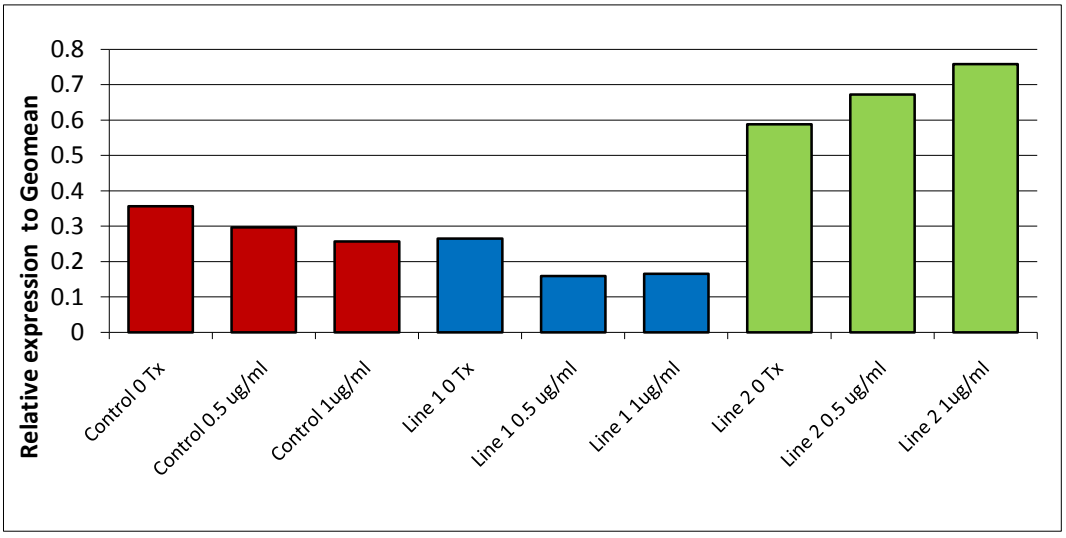


Figure 47: The expression of *Elf5* in the control and transfected mouse trophoblast stem cell lines. Line two shows a successful over expression of *Elf5* at the RNA level compared to the control and line 1. Expression is shown relative to the geometric mean of Gapdh, β-actin and β-tubulin.

The expression of *Cyr61, Calca, Sox2, Keratin7, Secretin, Wdr40B, Zic3, 1700112c13*, *Spi8* and *Gm784* (some of the predicted Elf5 targets) were analysed next. Of the genes analysed, 6/10 were regulated as predicted (*Cyr61, Calca, Kertain7, Spi8, Secretin* and *Gm784*). *Cyr61* was down regulated upon the loss of *Elf5* and it was up regulated when Elf5 is over expressed indicating it is a direct target of Elf5. 4/10 of the genes analysed (*Sox2, Wdr40B, Zic3* and *1700112c13*)

did not behave as predicted indicating some other protein may be involved in its regulation.

Sox2 relative expression levels were similar to the untransfected control (around 0.2) and to those of *Elf5*. Figure 48 below shows *Sox2* expression relative to the untreated/ no OHT sample. We would expect *Sox2* to be up regulated upon the addition of 4-hydroxytamoxifen. Interestingly it was down regulated in both cell lines. This indicates there is a complex network of interactions between genes in the *Elf5* transcriptional network.

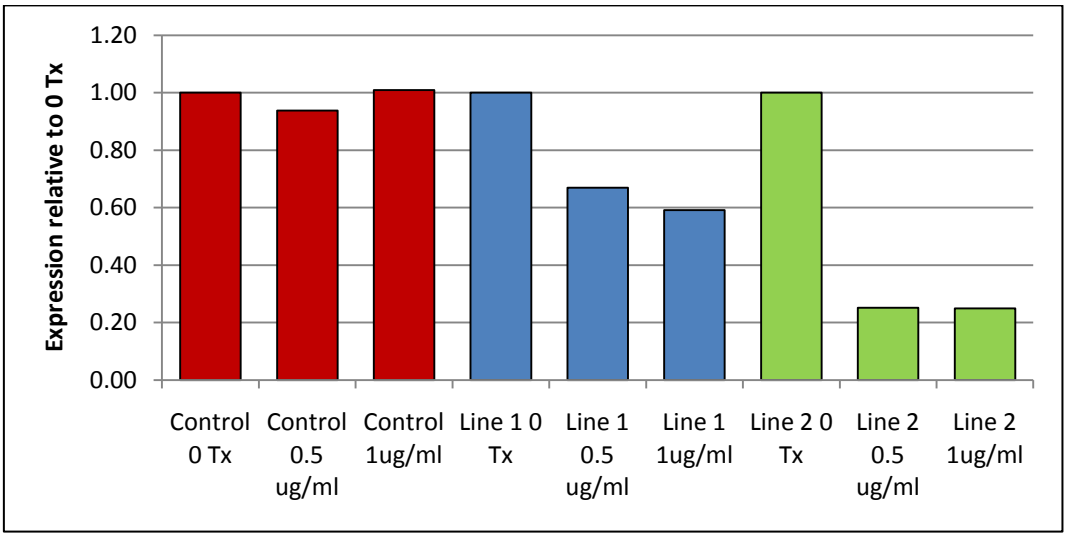


Figure 48: changes in the expression of *Sox2* upon the addition of 4-hydroxytamoxifen (OHT) and therefore the over expression of Elf5. Results were normalised to the geometric mean and then to the 0 OHT control. *Sox2* was down regulated when Elf5 was over expressed (addition of OHT) compared to the no OHT treatment. There was no change in *Sox2* expression in the control.

Calca was expressed at quite low levels in mouse TS cells. Compared to the control, Line 1 has similar expression levels. For an unknown reason *Calca* was expressed at even lower levels in line 2. See Figure 49.

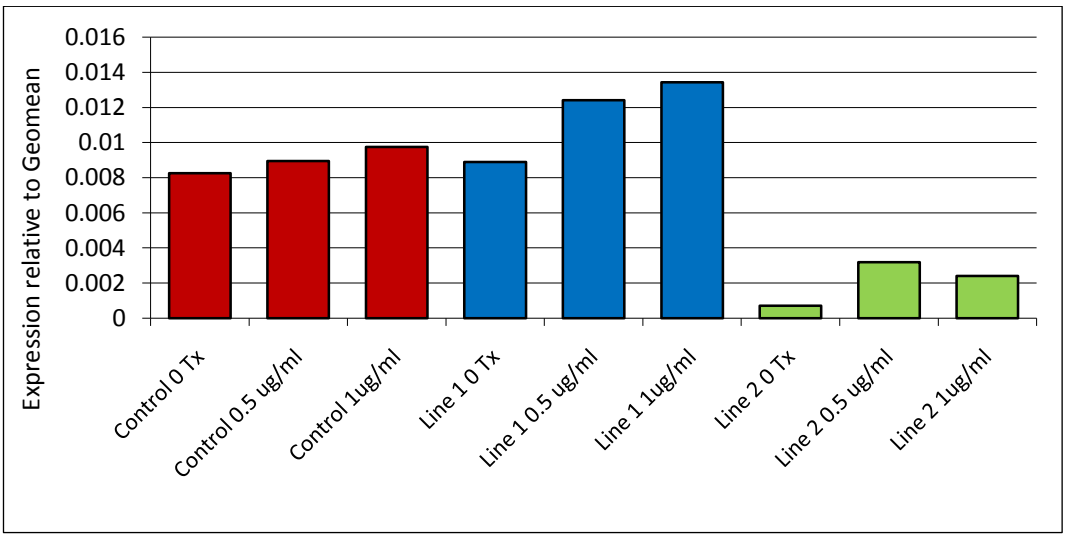


Figure 49: relative levels of *Calca* expression in the different trophoblast stem cell lines compared to the geometric mean of three house keepers. *Calca* was expressed at low levels in mouse trophoblast stem cells.

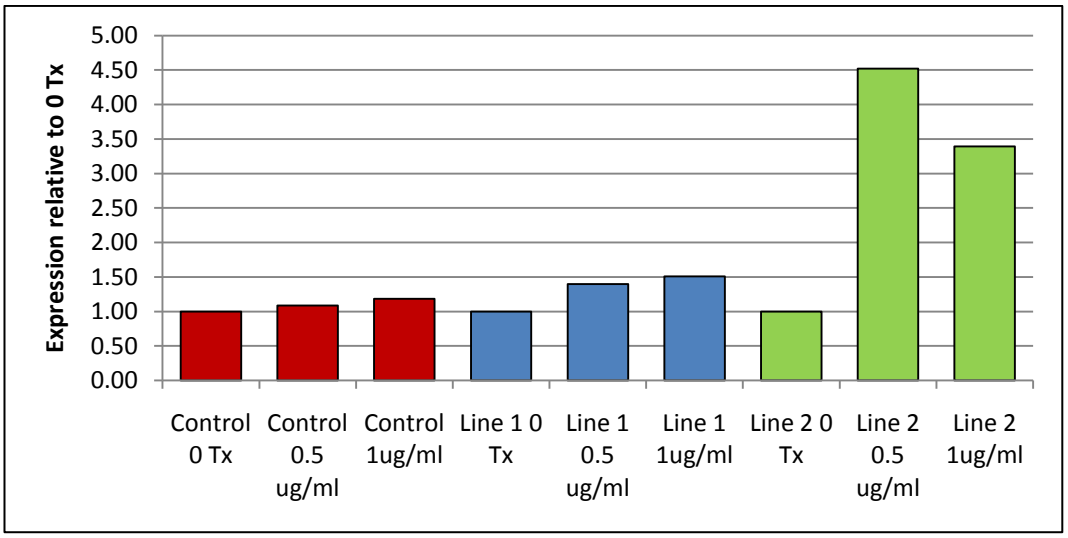


Figure 50: *Calca* expression shown relative to the no 4-hydroxytamoxifen (OHT) control for each trophoblast stem cell line. Upon the over expression of Elf5 (addition of OHT) *Calca* was up-regulated as expected.

When the control/ 0 OHT was normalised to 1.0 there was a large up-regulation of *Calca* upon the addition of 4-hydroxytamoxifen in line 2, see Figure 50. There was only slight up-regulation in line 1. So even though *Calca* was expressed at quite low levels in TS cells it is still of interest as an Elf5 target gene.

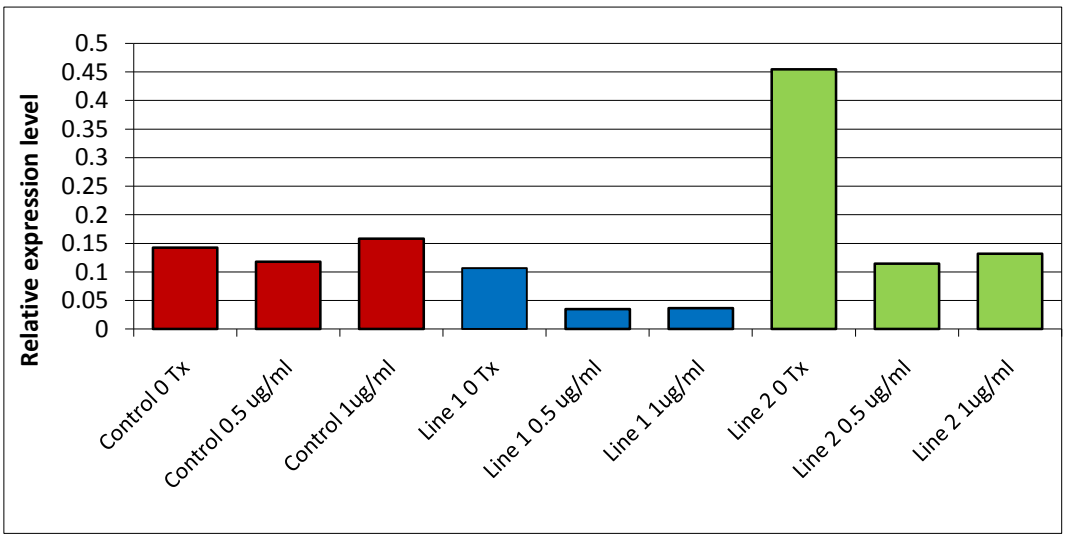


Figure 51: secretion expression with Elf5 over expression in mouse trophoblast stem cells. Secretin was down regulated as expected with Elf5 over expression (the addition of 4-hydroxytamoxifen). Expression was shown relative to the Geometric mean of three housekeeping genes.

Basal *Secretin* expression levels in line 2 also differed compared to the basal control levels (almost 5 times greater), see figure 51. Secretin was downregulated when Elf5 was over expressed compared to the 0 OHT control.

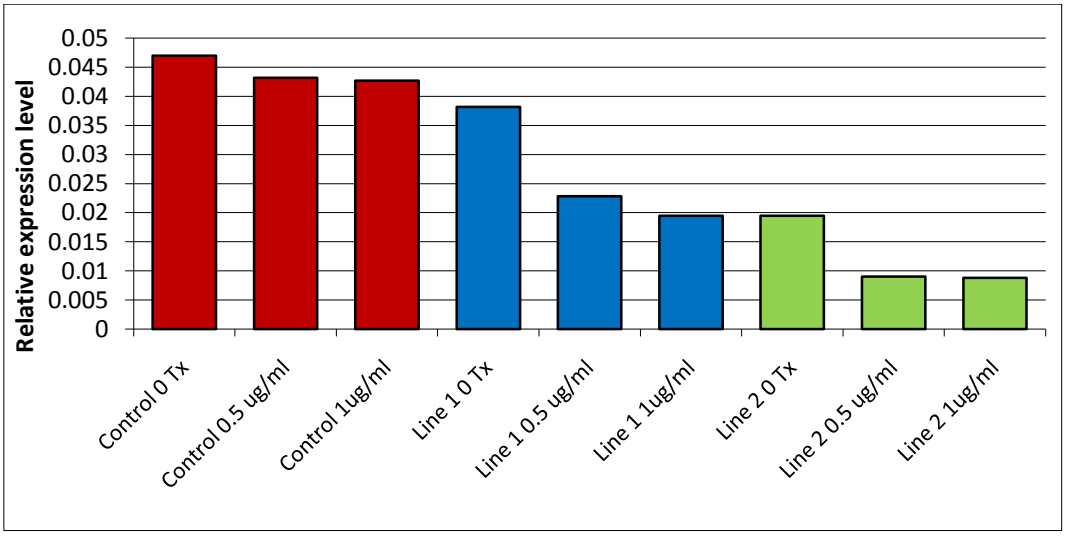


Figure 52: Wdr40B expression in mouse trophoblast stem cells with Elf5 over-expression. Wdr40B was down regulated upon the addition of 4-hydroxytamoxifen instead of up-regulated as expected. Expression is shown as levels relative to the Geometric mean of three housekeeping genes.

Wdr40B is a gene that does not behave as expected. Theoretically it should have been up-regulated upon the over-expression of Elf5. The levels of expression in line 2 also differ to the control, see Figure 52.

The addition of 4-hydroxytamoxifen did seem to induce the over expression of Elf5. But there was also more going on, some of the genes did not behave as expected and the levels of the genes varied compared to the control. As this was only a preliminary experiment and done in singlicites, this experiment will need to be repeated in triplicate whilst using only 1 µg/ml of OHT in future studies.

5.2.4.4 Analysis of genomic DNA of other clones

Other lines were obtained from the third transfection using puromycin resistance as a selection marker. These lines would not necessarily have the Elf5 mo Vp16 ER pyCAGiZeo plasmid inserted into their genome as it was a co-transfection with the puromycin cassette on another plasmid. Therefore it was necessary to do a PCR on the genomic DNA of these cells lines to ensure they have the plasmid of interest inserted into their genome. Once again a region was amplified by using the primers designed to the zeocin region of the plasmid. See Figure 53 below.

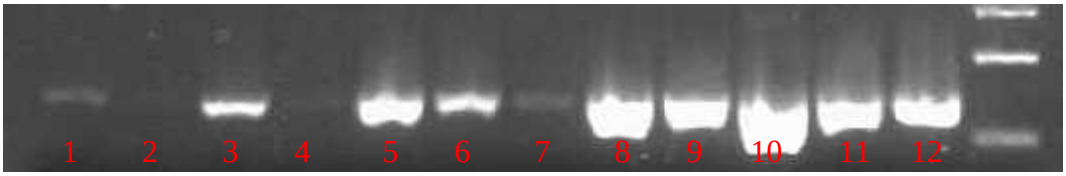


Figure 53: screening of other lines for insertion of Elf5 mo VP16 ER pyCAGiZeo plasmid into the genome. 1= MQ, 2= untransfected TS cells, 3-11 = lines 3-11, 12= positive control plasmid diluted 1:10,000. Lines 4 and 7 did not contain the plasmid inserted into their genome. The other cell lines analysed did contain the Elf5 mo VP16 ER pyCAGiZeo plasmid into their genome.

Lines four and seven did not have the plasmid inserted into the genome, they only had the puromycin resistance cassette. Lines 3, 5-6, 8-11 did have the plasmid inserted into their genome. These lines were grown up in 6-well plates and then frozen down according to the protocol outlined in 2.1.1.4 for future use and analysis.

5.2.5 Summary

The over expression of Elf5 was considered important as it provided more vital information regarding Elf5 as a transcriptional regulator. The initial Elf5-ERT2 plasmid did not seem optimal so the transcriptional activation domain of the herpes simplex VP16 virus was inserted. The VP16 domain had been used

successfully by Niwa et al,. (2005) and others to help over express their gene of interest. The addition of the VP16 domain did have its issues. Firstly the size of the insert was relatively small so it made it very difficult to obtain after repeated digestion and gel purification steps. And then it would not ligate into the Elf5-ErT2 plasmid. Once the decision had been made to insert the VP16 fragment into the p-GEM-T-EASY plasmid things began to work. All the subcloning procedures were very inefficient with only a few positive results at each step. Finally a clone was obtained with the correct insert and it was sequence verified.

Two stable cell lines were generated from the first transfection. This was extremely inefficient but I have been told that it can occur. The initial problem generating the lines began with the zeocin selection. As zeocin was a slow acting antibiotic the cells became far too confluent before the antibiotic had time to act on the untransfected cells. The concentration had to be increased from 200 µg/ml to 500 µg/ml. Eventually this caused a large amount of cell death and probably killed some colonies that should have been resistant to the antibiotic too. Once the antibiotic concentration was decreased back to 200 µg/ml some colonies started to appear. 58 colonies were picked but only two became established. This was probably due to inexperience with the picking technique.

Of the two lines that were obtained, they were shown to express the plasmid and produce the protein by immunocytochemistry. The cells express the protein but not all of it went into the nucleus upon the addition of 4-hydroxytamoxifen. Increasing the concentration of OHT would not help as it would affect the controls as OHT also binds to *Errβ* a gene involved in TS cell maintenance.

Although only a small amount of protein went into the nucleus, it did have an effect. Firstly the levels of *Elf5* were examined compared to the control. The *Elf5* levels were the same as the control in line one but 2-3 times higher in line 2. The TS cell marker genes *Eomes* and *Cdx2* were expressed in line one and two at the same levels as the control line and they were not up or down regulated upon the addition of 4-hydroxytamoxifen. This was satisfying as we have previously shown that *Eomes* and *Cdx2* were not affected by changes in *Elf5* expression.

The preliminary results obtained were not as expected. Some of the trends differed to the knockdown data and to complicate matters more the expression levels of the genes in the lines varied compared to the controls. Some of the genes behaved as expected, *Cyr61, Calca, Secretin, Keratin 7, GM784 and Spi8*. They show trends that correlate to the knockdown data. For example *secretin* was up regulated when *Elf5* was knocked down and it was down regulated when Elf5 was converted to an activator. But the levels of *secretin* expression differed to the control levels. This may have been due to leaky expression from the plasmid or some other contributing factor. *Sox2* was one of the best targets that we had identified and it was not up regulated as expected when Elf5 was over expressed. Instead it was down regulated. This could indicate that *Sox2* is not a direct target of Elf5, but that Elf5 regulates another protein involved in *Sox2* regulation. *Sox2* was expressed at the same levels to the control. This preliminary data indicates that there is a very complex network of interactions taking place.

Elf5 is a transcription factor and thus may normally work as a direct activator of some genes and a repressor of others, depending on other cofactors that bind to the target genes. In cases where Elf5 normally works as a repressor, the over expression of our Elf5-VP16 may well mimic knockdown. The removal of the repressor via knockdown is the same as the addition of an activator overcoming repression. For genes where Elf5 normally acts as a direct activator over expression of Elf5 may have no effect if endogenous levels in TS cells are high enough. This is why VP16 was added, to boost any effect. The likely reason that the expression of *Sox2* goes down is because the effect of Elf5 on *Sox2* is not direct. If Elf5 was an activator of *Sox2,* expression should have gone up, this was not the case. The most likely explanation is that Elf5 normally represses a repressor of *Sox2*.

More experiments need to be carried out but they fall outside the scope of this thesis. Additional cell lines that contain the Elf5-mo VP16 ERpyCAGiZeo plasmid integrated into their genome have been obtained but they have not yet been analysed.

6 Chapter Six: Cellular changes to mouse Trophoblast stem cells upon *Elf5* knock down

6.1 Overview

Chapter three to five described the genetic effect of the loss of *Elf5*, here we now investigate the cellular effect of the loss of *Elf5*. To assess the cellular changes that the trophoblast stem cells undergo when *Elf5* is knocked down the morphology, differentiation, proliferation and apoptosis of these cells were studied. When *Elf5* is knocked out *in vivo* the extra embryonic ectoderm (ExE) is missing, assumed to be due to the trophoblast being unable to maintain the trophoblast stem (TS) cells in an undifferentiated state (Donnison et al., 2005). The TS cells that reside in the ExE, and its precursor the polar trophectoderm are thought to have precociously differentiated into ectoplacental cone (EPC) cells; which leads to an absence of the ExE by E6.5. It would be interesting to see what happens to the TS cells *in vitro* when *Elf5* is knocked down since this study cannot be done *in vivo*.

The major technique used currently to examine proliferation, changes in DNA content and apoptosis has been flow cytometry. Flow cytometry is a useful technique that can provide information about a cell's size, granularity, internal complexity and other properties when the cells are labelled with fluorescent markers. Flow cytometry measures properties of single cells in a fluid suspension as they pass one at a time through the sample injection port (SIP). Large numbers of cells can be measured as the flow rate can be up to 2,000-5,000 cells per second. Flow cytometers measure light signals produced by the cells as they pass the 488 nm argon ion laser. Detectors are focused in line where the cell passes the light beam and measures forward scatter (FCS), side scatter (SSC) and there are also three fluorescent detectors for different wavelengths (green, yellow and red). Forward scatter is proportional to the cells size and side scatter provides information about the cells granularity and internal complexity. Cells labelled with Fluorescent markers and antibodies can provide information about the cell cycle, proliferation, viability and much more.

Before each assay the machine must be optimised with control cells. This is achieved by adjusting the voltage settings for FSC, SSC and the fluorescent channels until the unlabelled or negative control cell population is in the 10^0-10^1 area of the histogram. A positive control must also be used to ensure there is a shift from the 10^0-10^1 area to above 10^1 and that the assay is in fact working successfully.

6.2 Results

6.2.1 Effect of *Elf5* Knockdown on Cell Morphology

The first change to be examined upon *Elf5* knockdown was morphology. No morphological changes had been observed after 48 hr of *Elf5* knockdown. Due to this, changes in morphology were investigated 5-6 days after *Elf5* knockdown. Although *Elf5* siRNA would have been diluted out after this period we were hoping to see some downstream effects from the knockdown of *Elf5*. Another reason for examining morphology after 5-6 days is that upon the removal of growth factors, differentiated giant cells do not begin to appear for 4 days (Erlebacher et al., 2004). Morphology was analysed by staining the cells with Phalloidin and Hoechst 33342. Phalloidin-TRITC binds to F-actin subunits. Actin is abundant in the cell membrane and can therefore be used to visualise the cell membrane and boundaries when excited at 532 nm. Hoechst 33342 is a nuclear dye, and fluorescence is enhanced upon binding to the minor groove of double stranded DNA. The use of phalloidin and H33342 together allows the visualisation of the cell nucleus and the cell membrane which provides information about the cells morphology and size. The protocol was followed as in 2.2.1.

6.2.1.1 Optimisation of Phalloidin concentration

To optimise for phalloidin concentration two different concentrations of phalloidin were tested. The recommended range was 1-20 µg/ml so 20 µg/ml and 5 µg/ml of phalloidin were tested. Both concentrations gave good staining. The first time this assay was carried out the cells were far too confluent by 5 days. The cells were growing on top of each other and it was hard to distinguish

individual cells. I decided the cells needed passaging 2-3 days after the initial transfection. Two days after the knockdown of *Elf5* all cells were lifted by trypsinisation and the remaining cells were scraped to prevent the loss of any giant cells that had formed as giant cells tend to be more resistant to trypsinisation.

6.2.1.2 Problems with morphology

The TS cells had been grown in activin preceding these morphological experiments. It was noted that when the cells were grown in continuous culture with activin they had changed morphology compared to growth in conditioned media. The cells did not grow in tight colonies and the cells seemed very pointed. Also upon the removal of growth factors the cells did not dramatically increase in size and form giant cells as expected. Figure 54 shows cells stained with phalloidin and H33342 when grown in activin and after growth factor removal. These cells had been grown in activin for longer than two months.

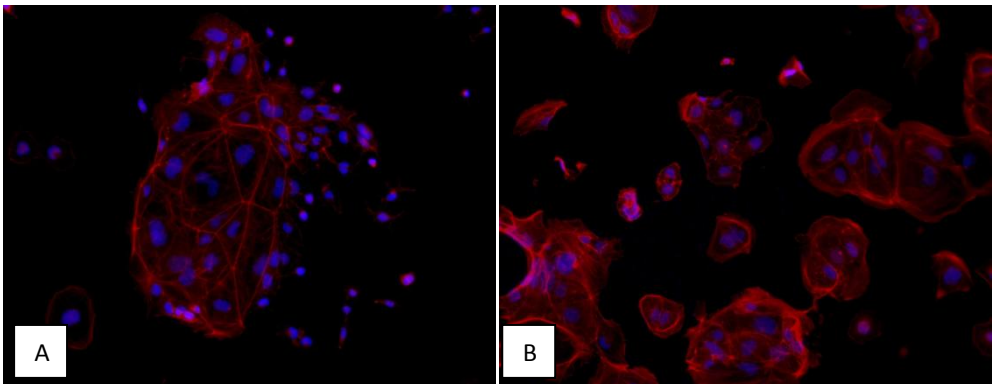


Figure 54: Non treated TS cells grown in the presence of activin (A) and cells with growth factors removed for 5 days (B). Note the appearance of the spiked cells in the no treatment cells and the cells do not increase in size upon the removal of growth factors. 10x magnification.

Although according to Erlebacher et al., (2004) activin can replace conditioned media for the culture of trophoblast stem cells, some morphological changes are apparent. Morphological changes were observed within a few weeks of culture in activin compared to those grown in conditioned media. The cells seemed to lose their tight epithelial boundaries and appeared to grow as single cells rather than in colonies. The cells were more pointed compared to the rounded cells observed when cultured in conditioned media. The cells did not differentiate into

giant cells or syncytiotrophoblast cells, had an altered appearance. Cells grown in activin for 1.5 months in contrast to those grown in conditioned media for 1.5 months can be seen in figure 55 below.

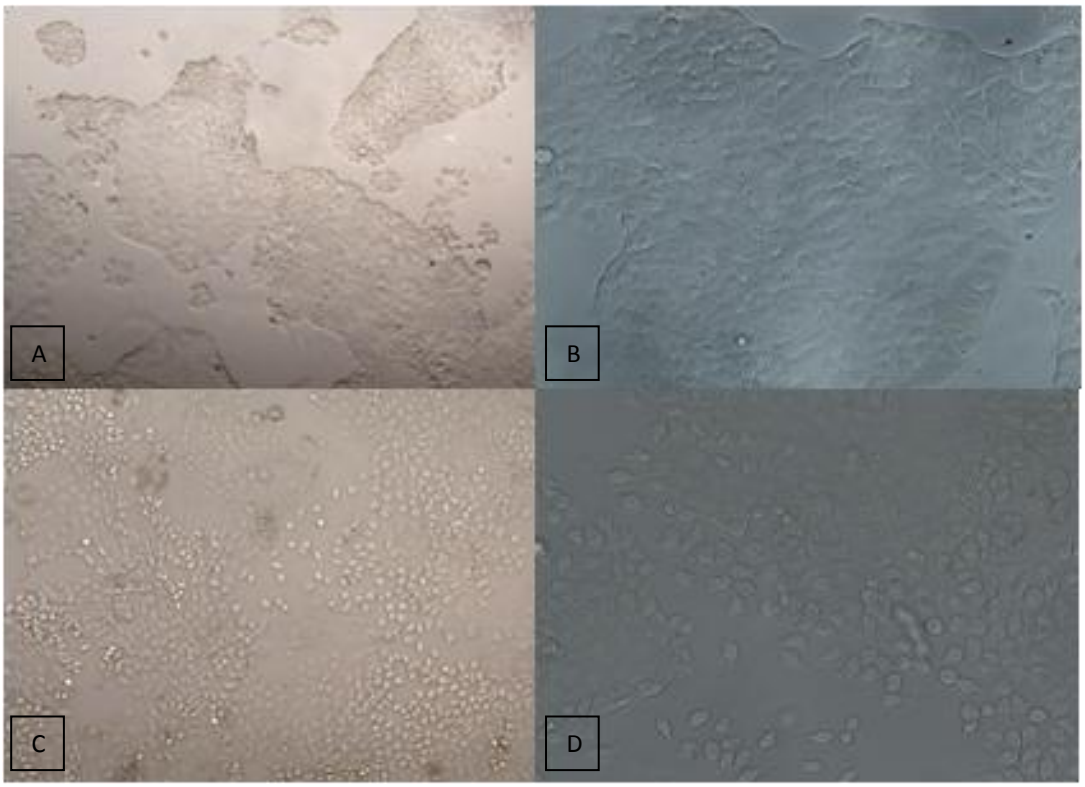


Figure 55: Cells grown in activin for 1.5 months compared to those grown in conditioned media. Cells grown in CM at 10x magnification (A), and at a 20x magnification (B). Colonies with tight boarders are clearly visible. TS cells grown in activin for 1.5 months at 10x magnification (C) and at 20x magnification (D). The spiked appearance of the cells can be seen.

In the short term there is no change in gene expression of TS cell marker genes (see Figure 7). Expression was not analysed after long term culture in activin. And therefore the cells could still be grown in activin in the short term for gene expression studies but cannot be used as indicators of changes in morphology.

6.2.1.3 Changes in morphology upon *Elf5* Knockdown

As I was concerned with the morphological appearance of the TS cells after preliminary experiments I decided to change back to using conditioned media instead of activin. Theoretically using activin instead of conditioned media was better as it was a more controlled media. The levels of growth factors can vary in each batch of conditioned media. But there was obviously something extra in the conditioned media that the TS cells required. *Elf5* was knocked down as outlined

in 2.2.1.1 and the morphology was examined after 6 days of *Elf5* knockdown by the procedure explained in 2.2.1.2-2.2.1.4.

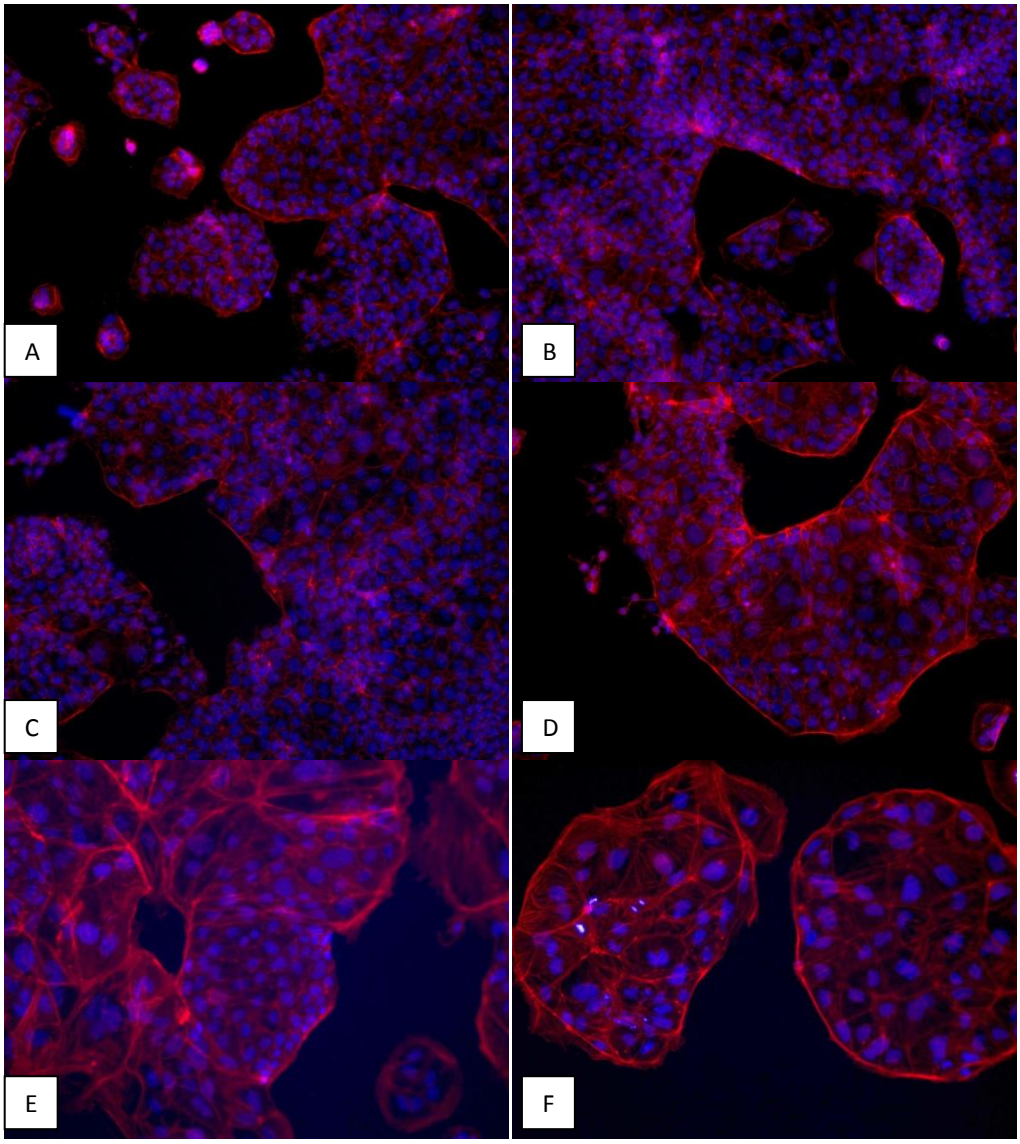


Figure 56: Morphological changes to TS cells upon *Elf5* knockdown. No Treated cells (A) Si negative control (B) 337 Elf5 knockdown (C) 733 Elf5 knockdown (D) No Growth factors (E & F) All pictures are a 10x magnification. The blue nuclear dye is H33342 and the red dye is phalloidin which binds to actin filaments in the cell membrane.

When the cells were grown in conditioned media the cells had tight borders and grew in TS cell colonies, see Figure 56-A. There was no change in morphology with the si negative control (B) compared to the no treatment control (A). 6 days after *Elf5* knockdown with the 337 siRNA there was possibly an increase in the number of larger cells (C). 6 days after *Elf5* knockdown with the 733 siRNA there was a more definitive increase in the number of larger cells (D). The cells were

not fully differentiated as upon the removal of growth factors (E,F), but they did look different to the no treatment and si negative control cells. Upon the removal of growth factors the cell size increased dramatically and the cytoplasmic volume also increased. These results were qualitative as there is no quantitative value assigned to morphological appearance. But these results were confirmed by analysing DNA content (see 6.2.2).

6.2.1.4 RNA isolation and Real time PCR to confirm *Elf5* Knockdown

To ensure *Elf5* was in fact knocked down during this morphology as well as the proliferation experiment (they were carried out on the same knockdown run) one well of TS cells which had been transfected was kept for RNA isolation. The cells were harvested 48 hr after the knockdown of *Elf5*.

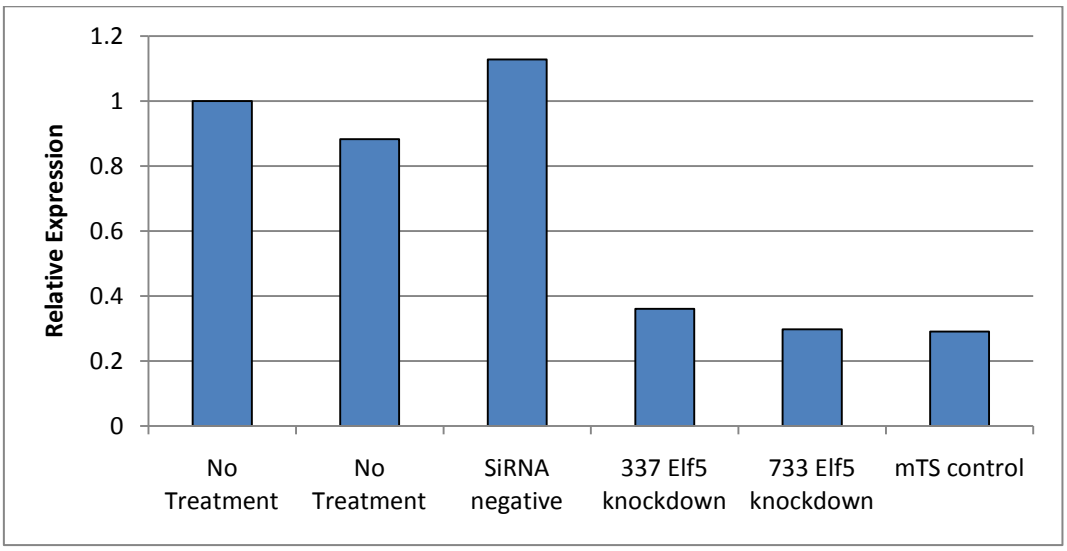


Figure 57: Percentage knockdown of Elf5 (337 and 733) for both the proliferation and morphology experiments. Expression is relative to the no treatment control. The mTS control was cells grown for 48 hr without growth factors.

A 70 % knockdown of *Elf5* was obtained during this experiment, this level of knockdown was sufficient to observe the downstream effects in terms of morphology.

6.2.2 DNA content

To provide further evidence of the state of the cell when *Elf5* is knocked down the DNA content of a cell can be measured by staining with propidium iodide (PI). Propidium iodide is a fluorescent compound that binds stochiometrically to nucleic acids so the amount of PI bound in the cell and therefore its fluorescence is proportional to the amount of DNA in the cell (Riccardi and Nicoletti, 2006). PI can therefore be used to distinguish between 2N, 4N and 8N or greater DNA contents. A 2N DNA content represents cells that are quiescent or in the G1 phase of the cell cycle. A 4N DNA content represents cells that are in the G2/M phase of the cell cycle. The synthesis phase of the cell cycle is between 2N and 4N. When the growth factors are removed TS cells endoreplicate to form giant cells. They can be distinguished from normal TS cells (2N and 4N) by the DNA content in the cell. Differentiated TS cells have a DNA content of 8N or greater (refer to section 1.3).

The cell must be permeabilised before the addition of PI as normal healthy cells can exclude PI. PI has been used previously to study the differentiation of TS cells upon the removal of growth factors (Tanaka et al., 1998) (see histogram below in Figure 58) and the addition of differentiation inducing agents (Yan et al., 2001).

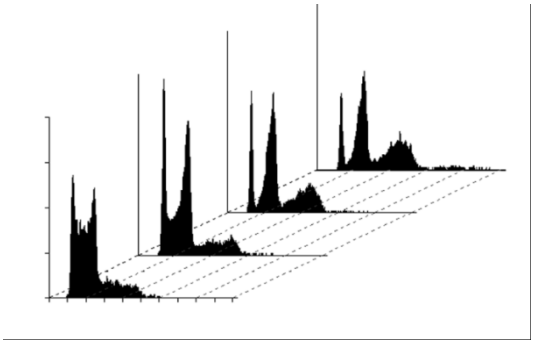


Figure 58: PI staining for DNA content of TS cells by Tanaka et al., (1998). Front histogram are untreated cells, moving back are 2,4, and 6 days after growth factor removal. There are three main peaks, the first is the 2N DNA content, second is the 4N DNA content and third is the 8N or greater peak. Note the 8N peak increases as the cells differentiate into giant cells when the growth factors are removed.

6.2.2.1 Optimisation of the FACS for DNA content

Before analysing DNA content of the TS cells upon *Elf5* knockdown the FACS had to be optimised. For optimisation $1x10^6$ untreated TS cells, differentiated TS cells

and mouse embryonic fibroblasts (MEFs) were grown up and fixed in 70 % ethanol over night. MEFs were used as a control cell line as they do not endoreplicate. They would only have a DNA content of 2N and 4N for non dividing and dividing cells respectively. The cells were stained with PI by the protocol outlined in 2.2.2.2-2.2.2.3. DNA QC particles (BD) were used for verification of instrument performance and quality control of the FACS before DNA analysis. These particles ensured there was a good separation between the G1, S, and the G2/M phases of the cell cycle. DNA staining by PI was measured in channel 3 (FL3, bandpass > 600 nm) with linear amplification. The MEFs were analysed for 2N and 4N DNA content. The histogram is shown below in Figure 59. M1 was a 2N DNA content and M2 was a 4N DNA content, M3-M4 was a DNA content greater than 4N. The small amount of cells with a DNA content of 8N were due to the heterogeneity of the embryonic fibroblast cell populations as these MEFs were only passage 2-3 of primary cells. The majority of the cells in this sample were in the M1 region which represents the 2N/G1 stage of the cell cycle.

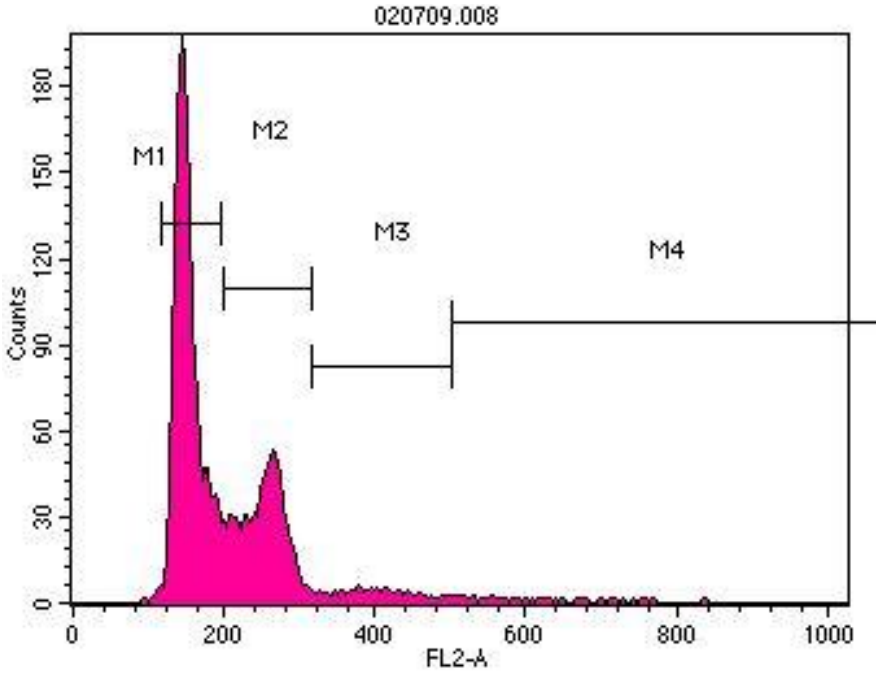


Figure 59: Mouse embryonic fibroblast (MEFs) cells used as a control for 2N and 4N DNA content. These cells do not endoreplicate so can be used as a control. The M1 peak in a 2N DNA content and the M2 peak is a 4N DNA content. The small peak in the M3 region is due to contaminating cell lines as MEFs are a primary cell line at an early passage number.

6.2.2.2 The Effect of *Elf5* Knockdown on DNA content

Once the FACS was optimised *Elf5* was knocked down as in 2.2.2.1 and the cells were cultured for 6 days upon *Elf5* knockdown. The cells were labelled by following the same protocol as above. The treatments were untreated, si negative, removal of growth factors and both of the Elf5 targeted siRNAs. Refer to table Table 21 in the appendices for raw data.

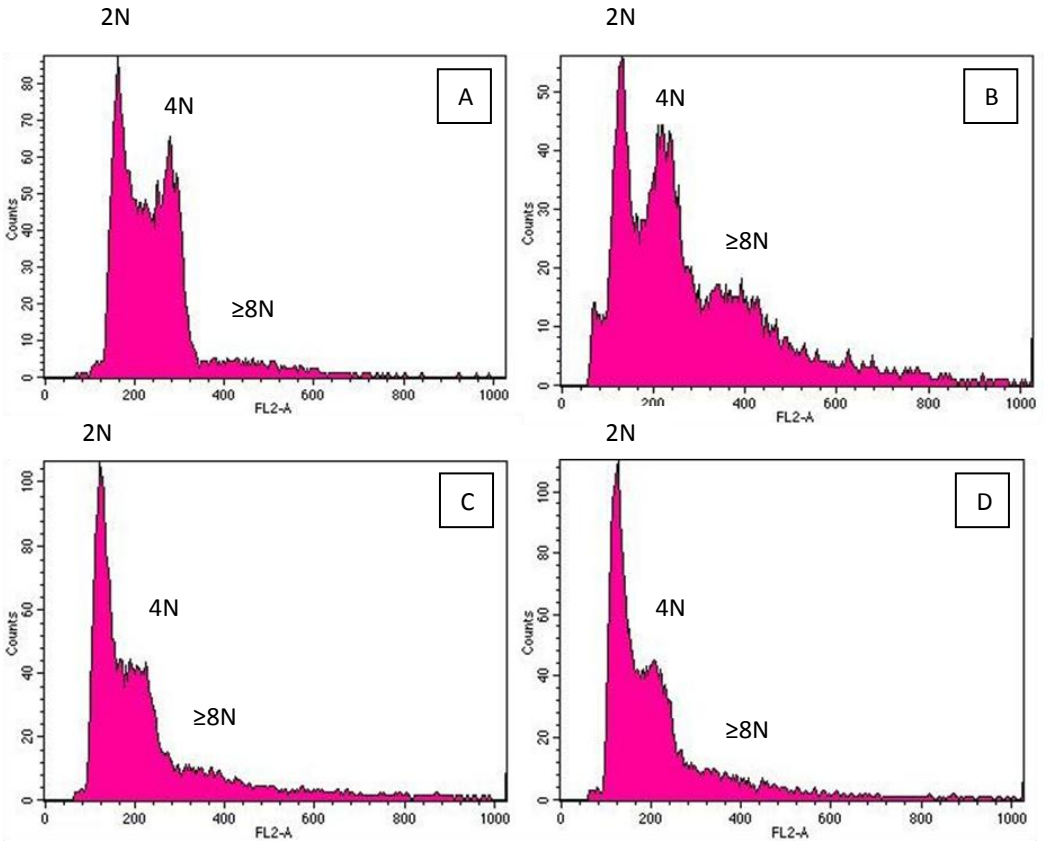


Figure 60: No treatment cells showing normal DNA content of 2N and 4N with little differentiation (8N peak) (A). TS cells differentiated for 6 days, notice the large ≥8N peak (B) 733 *Elf5* knockdown (C) and 337 *Elf5* knockdown (D), increase in ≥8N peak seen for both.

Figure 60 are some examples of the histograms obtained 6 days after the knockdown of *Elf5*. Figure A shows what the DNA state of TS cells grown under normal conditions. Note there was only a small peak of cells with a DNA content of greater than 4N. Also note the similarity to the first histogram in Figure 58. When the growth factors were removed the number of differentiated cells increased from 11 % in the no treatment to 35 % after 6 days differentiation. This value may have been larger as some differentiating cells within the 4N peak may be endoreplicating not dividing. There was an increase in the number of

differentiated cells when *Elf5* was knocked down. For the 337 *Elf5* knockdown the number of cells with a 8N or greater DNA content increased by 6 % and when *Elf5* was knocked down by the 733 siRNA this increased by 8 %. See Figure 61 for a bar graph of the difference in DNA content for the different treatments. Another interesting change to note was that the 2N and 4N population of 337 and 733 changed compared to the untreated control. The 2N DNA content increased and the 4N decreased. This could have potentially been a proliferative effect which was examined later by a different technique. This change was also seen in the si negative control.

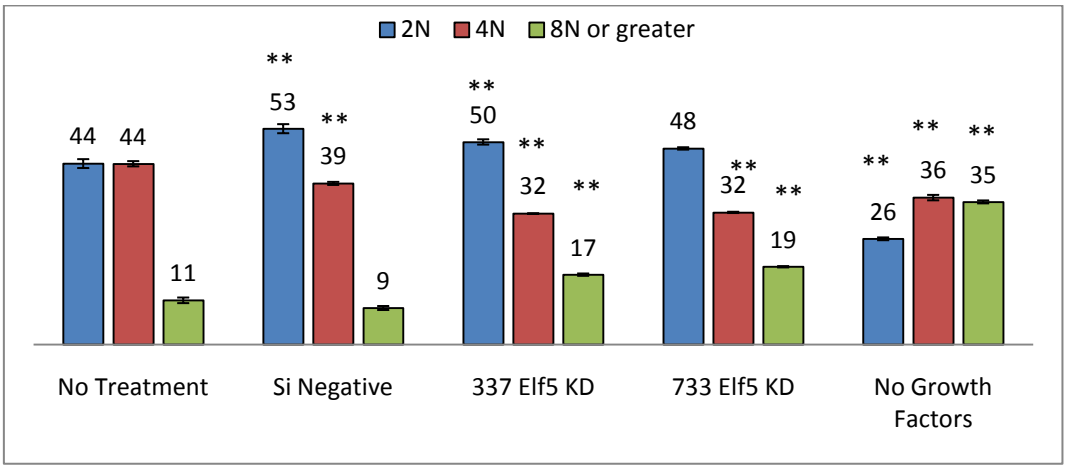


Figure 61: Changes in DNA content upon *Elf5* knockdown in mouse trophoblast stem cells. N=4. Note when the cells were transfected there was a decrease in the number of cells with a DNA content of 4N and an associated increase in the 2N peak. This was seen in the si negative as well as the *Elf5* transfections so this was not specific to the knockdown of *Elf5*. There was also an increase in the 8N or greater peak when Elf5 was knocked down. Significance compared to the no treatment control ** P<0.01

6.2.2.3 RNA isolation and Real Time PCR

Again to ensure *Elf5* had in fact been knocked down during the DNA content experiment one well of each treatment was kept for Trizol isolation 48 hr after the knockdown of *Elf5*. A 70 to 80 % knockdown of *Elf5* was achieved for this experiment relative to the no treatment control.

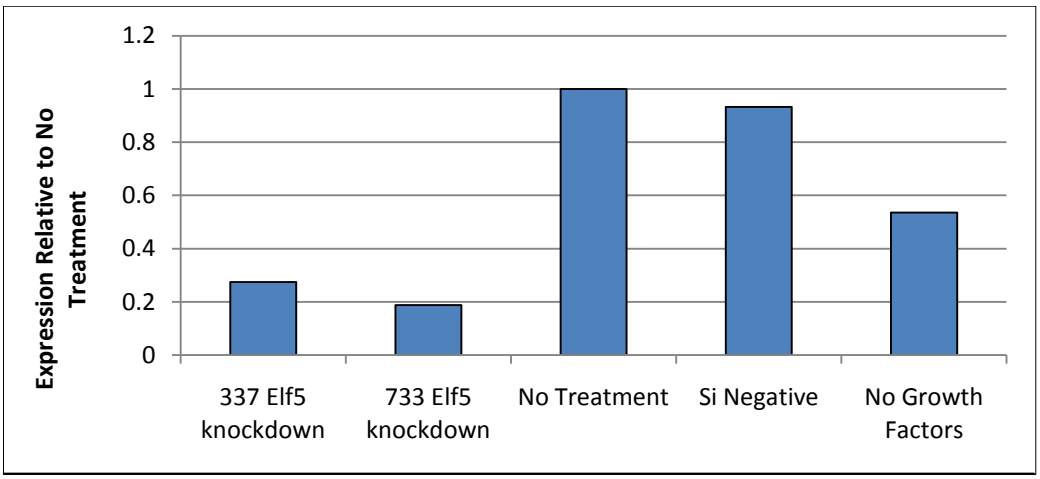


Figure 62: Knockdown of *Elf5* for the DNA content experiments, achieved greater than 80 % knockdown compared to the no treatment control in mouse trophoblast stem cells.

6.2.3 Proliferation

Elf5 is potentially a factor involved in the maintenance of proliferation in the trophoblast as in its absence the extraembryonic ectoderm cannot be maintained (Donnison et al., 2005; Rielland et al., 2008). A marker for proliferation is the phosphorylation of histone 3 at serine 10 that is initiated in late G2. It is at a maximum during metaphase and begins to diminish during anaphase of mitosis, being associated with chromosome condensation (Gurley et al., 1977; Hendzel et al., 1997). Antibodies to this marker are sensitive (see Figure 44) and can be combined with flow cytometry to determine the amount of proliferative cells in a given sample or treatment. As for morphology and DNA content the proliferation of the cells were examined 5 days after the knockdown of *Elf5.*

6.2.3.1 Optimisation on the FACS

Before the proliferation experiment could performed, the concentration of the primary antibody (rabbit antibody against H3 Serine 10 phosphorylation) needed to be optimised. Three different primary antibody concentrations were tested, 1:100, 1:200 and 1:500 along with a 1:1000 dilution of the secondary antibody which was an Alexa 488 donkey anti rabbit antibody. The optimal concentration was 1:200, a distinct second peak was observed with this concentration.

The instrument settings also had to be optimised with control samples. The negative control was the secondary antibody alone without the primary antibody to give the level of non specific binding. Mitomycin C was used as a negative control, it is a DNA cross linker so it prevents DNA replication. The positive control was cells treated with nocodazole. Nocodazole causes late G2 or mitotic arrest (Kwak et al., 2005), which is useful as a control as Histone 3 serine 10-phosphorylation occurs at the late G2/M phase of the cell cycle. The cells were treated with nocodazole and mitomycin C for 16 hr. The protocol for the analysis of proliferation is outlined in 2.2.3.

6.2.3.2 The Effect of Elf5 Knockdown on Proliferation

Elf5 was knocked down as in the other assays and the cells were analysed for this proliferative marker 5 days after transfection. The cells had to be passaged once during this treatment as they became too confluent.

Under normal conditions the percentage of mitotic cells in a population of TS cells is 4.7 %, see Figure 63. This was the total number of cells going through the late G2/M phase at the give time point. The cells were not selected by shaking to detach the mitotic cells. This level of proliferation seemed relatively low but we were only capturing unsynchronised cells at a certain time point. Upon 50 ng/ml nocodazole treatment the percentage of mitotic cells was significantly increased to 14.2 %. This concentration was saturating as 100 ng/ml nocodazole did not increase the number of proliferative cells. Mitomycin C treatment only decreased the number of mitotic cells to 2.6 %. The removal of growth factors decreased cells proliferation to 1.1 %. This was what was expected as the majority of the cells would not be going through the G2/M phase of the cell cycle. Instead they will be going through repeated rounds of DNA synthesis. The knockdown of *Elf5* did not have any significant effect on cell proliferation using this mitotic marker. Refer to table Table 19 in the appendices for raw data.

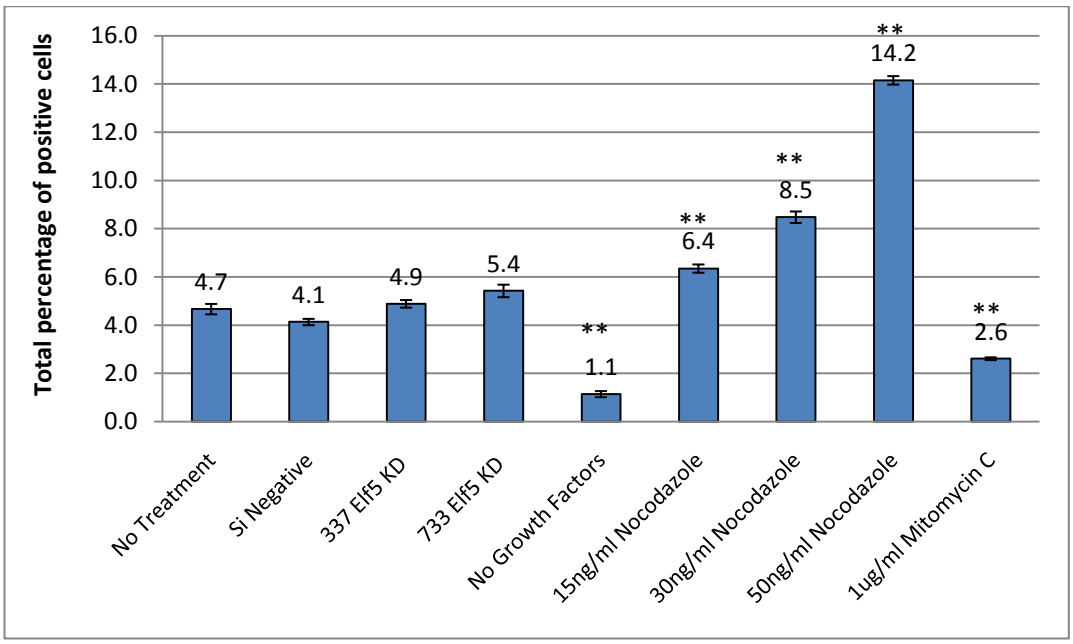


Figure 63: Levels of histone 3 serine 10 phosphorylation in different treatments which shows the number of mitotic cells in each treatment. N=5. Significance compared to the no treatment **p<0.01. There was no change in proliferation upon the knockdown of Elf5 (337 and 733).

6.2.4 Apoptosis

Apoptosis is programmed cell death that occurs in eukaryotic cells. Apoptosis is involved in many processes in a cell including disease, tumorgenesis and embryogenesis and can occur in response to stress, inflammation and infection. Apoptosis occurs in an orderly fashion which includes cell shrinkage, activation of caspases, loss of membrane integrity, DNA fragmentation and the formation of apoptotic bodies. These processes can be exploited and used as indicators of apoptosis (Vermes et al., 2000). Activation of DNases during apoptosis results in extensive DNA fragmentation in the cell. These DNA strand breaks can be used as an indicator of apoptosis by labelling the strand breaks with a fluorochrome. This is termed a TUNEL assay (Terminal deoxynucleotidyl transferase-mediated dUTP Nick end labelling). The 3'-OH termini of DNA double strand breaks can be labelled with BrdU when incorporated with BrdUTP by the terminal deoxynucleotidyl transferase (TdT) enzyme (see Figure 64 below). The strand breaks can then be detected by a BrdU secondary antibody conjugated to an Alexa 488 dye and measured by flow cytometry (Darzynkiewicz et al., 2008). Flurochromes other than BrdU are commonly used but BrdU increases the sensitivity of the assay by giving a four-fold higher signal (Vermes et al., 2000). A

drawback of this method is that DNA strand breaks can occur in other modes of death too. The incorporation of PI staining into this method allows the identification of cells that have a sub-diploid DNA content which is another marker of apoptosis. An APO-BrdU™ TUNEL Assay Kit was purchased from Molecular Probes™ (invitrogen) and was used in this section in combination with flow cytometry.

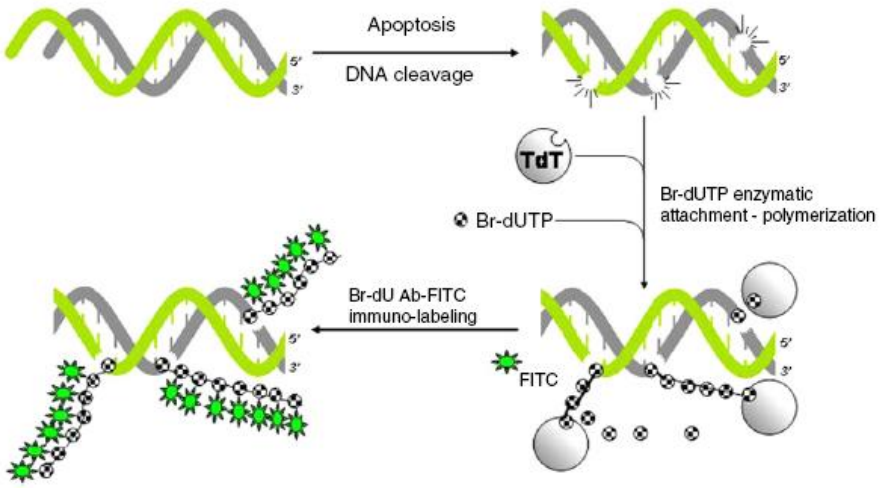


Figure 64: Schematic showing TdT incorporating BrdUTP into cleavage site of the DNA. An Fluorescently labelled secondary antibody to BrdU then binds to it (Darzynkiewicz et al., 2008). The number of fluorescing cells can then be determined by flow cytometry. Figure borrowed from Darzynkiewicz et al.,(2008)

6.2.4.1 Initial trial of the tunnel assay

Before knocking down *Elf5,* the assay must be optimised for the TS cells. In the first instance untreated cells were labelled by following the protocol outlined in 2.2.4.2. Both positive and negative control cells were provided with the kit. The positive control cells were a human lymphoma cell line treated with the DNA topoisomerase inhibitor camptothecin. Camptothecin induces apoptosis selectively during S phase of the cell cycle (Darzynkiewicz et al., 2008). The negative control line was the same cells untreated. The protocol provided with the kit worked well on the positive and negative control cell lines. There was no detectable apoptosis in the untreated TS cells, see Figure 65. But a negative result did not necessarily mean that there was no apoptosis, as the assay may have not been working on the TS cell line.

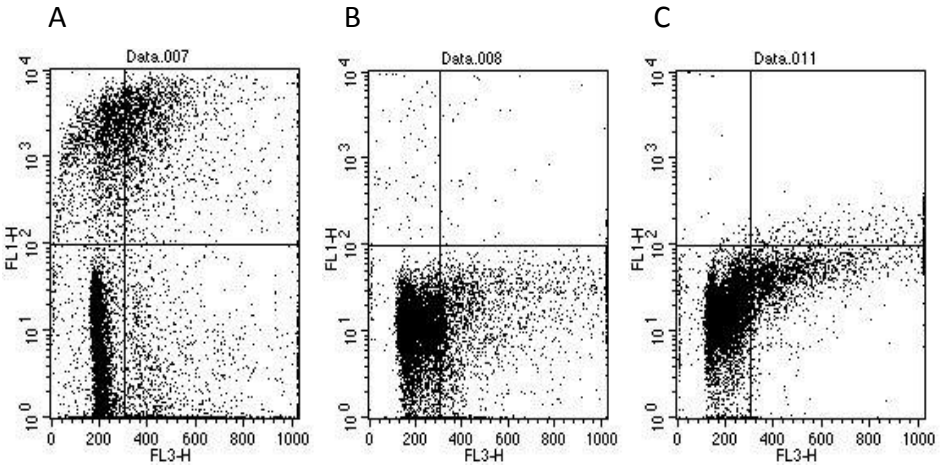


Figure 65: Positive control cells (A), Negative control cells (B), TS cells untreated (C). FL1 is green fluorescence which was BrdU labelling, FL3 is PI staining. A shift to 10^2-10^4 in FL1 indicates apoptotic cells. A shift into the 10^2-10^4 region on the Fl1 spectrum was seen in the positive control cell line and no shift was seen in the negative cell line.

6.2.4.2 DNase treatment of the TS cells

Due to the negative result, during the next assay the TS cells were treated with DNase for 10 min at RT. After the cells had been fixed and stored in ethanol overnight, the cells were washed twice with DNase I buffer and then resuspended in 100 µl DNase buffer. 5 units of DNase I was added and the cells were incubated at RT for 10 min. DNase induces DNA strand breaks and therefore can act as a positive control. The cells were then washed with wash buffer and the usual protocol was followed. No positive cells were obtained after DNase treatment. This indicated that either the DNase treatment wasn't working or the assay was not optimised for the TS cells. See Figure 66 below.

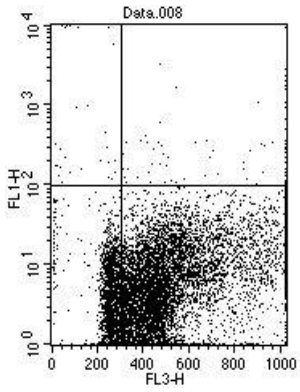


Figure 66: TS cells treated for 10 min at RT with DNase, there was no DNA strand breaks as there was no fluorescence observed at 10^2-10^4 in the FL1 channel.

6.2.4.3 DNase treatment of the negative control cell line

To make sure the DNase treatment was not the problem the negative control cell line provided with the kit was treated with 5 units of DNase for 10 min as above. The majority of the cells were positive for DNA strand breaks using this method (see Figure 67 below). This showed that the DNase treatment did work and that the assay conditions needed optimising for the TS cells themselves.

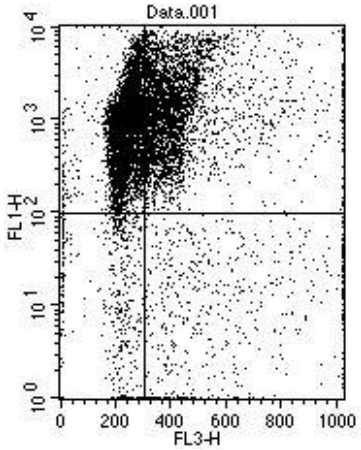


Figure 67: Treatment of negative control cell lines with DNase. Note all cells are now positive for DNA strand breaks as they have moved to the 10^2-10^4 region on the histogram.

6.2.4.4 Optimisation of assay conditions

According to the protocol supplied with the Tunnel assay the incubation times with the TdT enzyme may need to be optimised. The incubation time with the TdT enzyme for the control cells was 1 hr. The incubation time with the TdT enzyme was increased to 4 hr. During this assay some cells that were floating in the media from another experiment were included as another control. These cells turned out positive in the tunnel assay and so did the DNase treated cells. The untreated TS cells did shift into the positive area of green fluorescence but this level of apoptosis was far too high to be correct so the incubation time was decreased to 2-3 hr for the next optimisation experiment. A TdT incubation time of 3 hr was concluded to be the optimal incubation time as the majority (93 %) of the DNase treated cells were positive for apoptosis as you would expect in an efficient digestion and the untreated cells did not shift to give false positives. The 2 hr TdT incubation was not long enough as only 61 % of the DNase treated cells were positive for DNA strand breaks.

6.2.4.5 Knockdown of Elf5 and analysis of apoptosis

Elf5 was knocked down in the TS cells according to the protocol outlined in 2.2.4. As the time that the cells would be undergoing apoptosis due to the knockdown of *Elf5* was not known, apoptosis was analysed both 48 hr and 5 days after the initial transfection. To ensure *Elf5* was in fact knocked down RNA was isolated from an extra well 48 hr following transfection; Figure 68 shows the knockdown of Elf5 achieved for apoptosis.

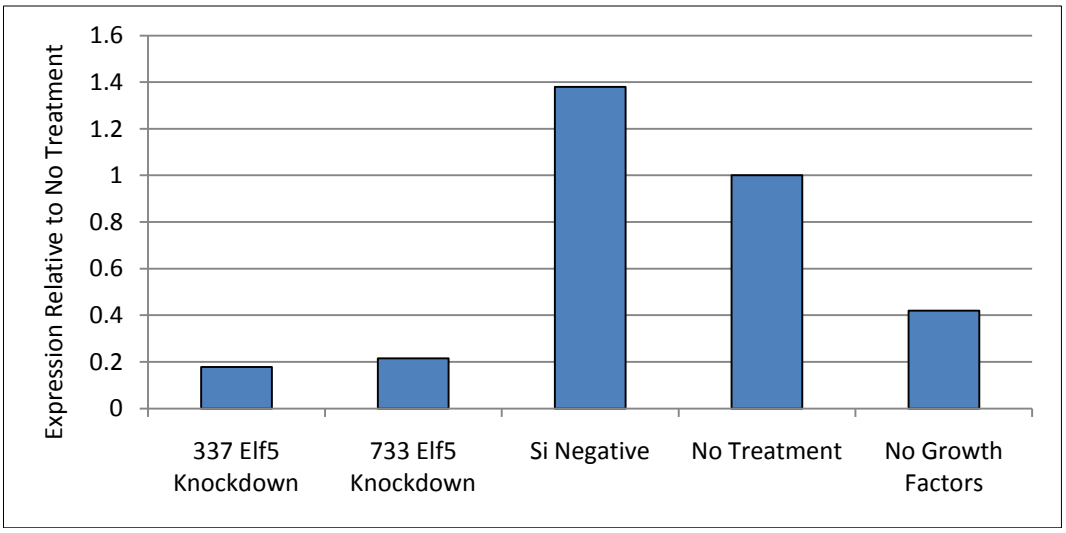


Figure 68: Elf5 knockdown for the apoptosis experiment in trophoblast stem cells. Greater than 80 % knockdown of *Elf5* was achieved in comparison to the untreated control for the apoptosis experiment. As expected when the growth factors were removed, *Elf5* was down regulated.

6.2.4.6 Apoptosis results

The tunnel assay was carried out on two separate days as there were far too many samples to do at once. The samples were incubated with the TdT enzyme for 3 hr as this time period was determined to be optimal by analysis of DNase I treated cells (see section 6.2.4.4). 48 hr following the transfection there were very few cells undergoing apoptosis in any of the treatments. The highest was 0.76 % in the si negative treatment. There was no difference between the samples in which *Elf5* was knocked down in and the no treatment. There was no increase in apoptosis when the growth factors had been removed from the TS cells for 48 hr either. After 5 days in culture the number of cells undergoing apoptosis upon *Elf5* knockdown increased significantly compared to the 5 day no treatment sample but this also occurred in the si negative control so it was not

specific to the knockdown of *Elf5*. There was no significant difference between the 5 day apoptosis levels between the si negative and the two samples with *Elf5* knocked down. There was a large increase in the number of cells undergoing apoptosis when the growth factors had been removed for 5 days. See Figure 69 below for a summary of the effect of *Elf5* knockdown and removal of growth factors on apoptosis. Refer to Table 20 in the appendices for the raw data.

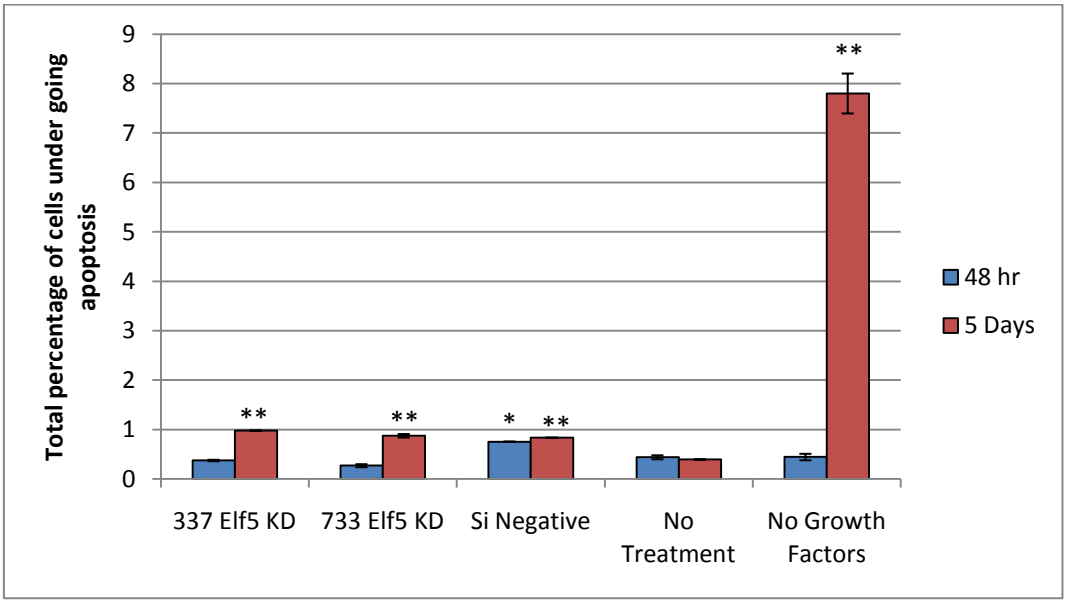


Figure 69: Total number of cells undergoing apoptosis in different treatments in mouse trophoblast stem cells. Only 0.7 % of cells were undergoing apoptosis in normal healthy TS cells. Significance difference compared to the no treatment control *p<0.05, **p, <0.01. N=2.

6.2.5 Summary

In addition to the changes in gene expression upon the knockdown of *Elf5*, the cellular changes in the TS cells upon the knockdown of *Elf5* were examined. Changes in morphology, DNA content, proliferation and apoptosis were studied as these are stem cell characteristics that could change due to the loss of expression of a critical gene.

The removal of growth factors as expected, had the most dramatic effect on the TS cellular characteristics (Cross et al., 2003; Hemberger et al., 2004; Yan et al., 2001). Changes in morphology were apparent 4-6 days after the removal of growth factors. There was an increase in cell size, especially the formation of a much larger nucleus and cytoplasm compared to TS cells. To coincide with the large nucleus there was an increase in the 8N or greater DNA content of the cell due to DNA endoreplication. There was also a decrease in proliferation due to endoreplication as the cells continue to synthesise their DNA without going through cell division. There was no change in apoptosis 48 hr after the removal of growth factors but there was a large increase after 5 days of differentiation.

The knockdown of *Elf5* caused a definitive increase in the number of larger cells compared to the untreated control by morphological observation. There was no change in morphology in the si negative control cells. The analysis of DNA content confirmed the increase in the number of larger cells. This change was not as large as the removal of growth factors, only some of the cells were differentiating. There was no increase in 8N DNA content in the si negative control. A decrease in the number of cells with a 4N DNA content was observed along with an increase in the 2N DNA content. This latter effect could have been a proliferative effect. The same change was seen in the si negative control. Although there was a change in the number of cells with a 4N DNA content upon the knockdown of *Elf5*, there was no change in proliferation. A change in proliferation could have possibly been seen at an earlier time point (for example

2-3 days after knockdown). The levels of apoptotic cells when *Elf5* was knocked down did not change in relation to the si negative control.

These results indicate Elf5 is likely to be involved in the prevention of differentiation of the TS cells into EPC/giant cells. Elf5 is not involved in apoptosis of the TS cells. Elf5 is not involved in cell proliferation at the time point studied but it could well be involved at an earlier time point.

7 Chapter 7: Discussion

7.1 Overview

The placenta is an essential organ for mammalian development as it mediates the interaction between the foetus and the maternal blood stream. At the early blastocyst stage of embryo development, two cell lineages can be distinguished, the inner cell mass (ICM) and the trophectoderm (TE). The ICM gives rise to the embryo proper and the extra embryonic endoderm, the TE the foetal part of the placenta. The polar trophectoderm which lies above the ICM receives signals from the ICM to form the extra embryonic ectoderm (ExE) and the ectoplacental cone (EPC) which can form all cells of the placenta (Simmons and Cross, 2005). Pluripotent trophoblast stem (TS) cells can be derived from the ExE and can be cultured *in vitro* in the presence of embryonic conditioned media, FGF4 and heparin (Tanaka et al., 1998). A DNA transcription factor Elf5, has been identified that is expressed in the ExE lineage and has been hypothesised to be required for TS cell maintenance (Donnison et al., 2005). Deane (2007) identified Elf5 target genes by RNA interference in TS cell lines by knocking down *Elf5*, followed by global gene expression analysis using an microarray (affymetrix) array. This gave a group of genes that were up or down regulated upon loss of *Elf5*. This work was continued here by investigating the effects of *Elf5* knockdown (via siRNA technology) on target genes after a period of 24 or 48 hr. *In situ* hybridisation was used to determine if the genes were expressed at the right time and in the correct place to be Elf5 targets. The initial ground work to get the over expression of Elf5 via the tamoxifen inducible system up and running has been done, with further work required in the future. The TS cell characteristics when *Elf5* was knocked down were also investigated with some interesting results.

7.1.1 Generation of *Elf5* knockdown

The aim of this section was to validate the Elf5 targets by repeating the knockdown of *Elf5* in mouse trophoblast stem cells. Two siRNAs had been identified by Deane (2007) as giving greater than 80 % knockdown of *Elf5* after 24 hr of transfection. In this study two different time points were investigated,

24 and 48 hr after the initial transfection. The changes in target gene expression were analysed by real time PCR.

The generation of a reasonable knockdown of *Elf5* was problematic and it was clear the system needed optimising. Optimisation was carried out using an Alexafluor fluorescent oligo. Different seeding densities, oligo and lipofectamine concentrations and forward and reverse transfections were tested. Flow cytometry was the best technique to determine transfection efficiency. Upon optimisation of the FACS it was established that greater than 90 % of the cells had been transfected with the fluorescent oligo when the cells were seeded in a 6-well plate. This was due to the fact that the cells did not clump in the middle of the well, as they did in 12 well plates making the cells more accessible to the siRNA/fluorescent oligo. When RNA was isolated after a transfection under the same conditions an 80-90 % knockdown of *Elf5* was achieved.

7.1.1.1 Replacement of Conditioned media with Activin

TGF-β and activin are key components of embryonic fibroblast conditioned media Erlebacher et al,. (2004). Studies (Erlebacher et al., 2004; Guzman-Ayala et al., 2004; Natale et al., 2009) have shown that the use of activin or TGF-β in place of conditioned media was possible and had no effect on the TS cells themselves. Due to these reports our group decided to change from using conditioned media to activin plus FgF4 and heparin to culture the TS cells. This was desirable as the levels of growth factors would not fluctuate. To ensure that there was no change in gene expression the expression of TS cell marker genes were analysed in four samples grown in activin versus four grown in conditioned media. The expression of *Elf5, Eomes, Bmp4, Cdx2* and *Fgfr2* was investigated. There was variability in expression between individual samples but when the levels were averaged they were similar. There was no significant change in expression of any of the marker genes. There was a 2x increase in Cdx2 when the cells were grown in activin but this was not statistically significant due to the variation of the individual treatments.

After culture in activin for over 1 month changes in the cell morphology were noticed. The cells did not grow as colonies, instead they almost grew as

individual cells. They also adopted a spiked appearance, they no longer looked like cobble stones. It was concluded that culture in activin was satisfactory for short term culture but was not optimal for the long term due to changes in morphology. Clearly there was something else in the conditioned media that the TS cells required, even if it was required at relatively low levels.

These findings are contrary to those of Erlebacher et al., (2004) who find that activin or TGF-β are a suitable replacement of embryonic fibroblast conditioned media for the long term culture of TS cells. Neither Erlebacher (2004) nor Natale (2009) mention any changes to the TS cell morphology when cultured in activin.

7.1.1.2 Real time data and whole mount *in situ* hybridisation results

The analysis of the spatial and temporal expression of the Elf5 target genes provided further evidence that these genes are in fact targets of Elf5. *Elf5* is expressed in the extra embryonic ectoderm from E5.5 and later in the chorion, the derivate of the ExE at E8.5 (refer to Figure 14). Genes that were expressed in this region during this time and were affected by the knockdown of *Elf5* were likely to be targets of Elf5.

7.1.1.3 Genes of Interest

Cyr61 codes for a cystine-rich angiogenic inducer 61, a secreted heparin-binding protein. It is encoded by a growth factor inducible immediate early gene which is rapidly activated when cells are stimulated with FgF, platelet derived growth factor and tumor transforming growth factor β (Mo et al., 2002). Results in this study indicated that *Cyr61* is expressed in the ExE at day 6.5 and later the chorion and allantois at day 7.5. It was also expressed in the EPC at this stage. Later on it is expressed in the giant cells surrounding the placenta and the spongiotrophoblast (E11.5) (O'Brien and Lau, 1992). *Cyr61* is also expressed in the developing embryo, in the smooth muscle and cartilage. The knockout of *Cyr61* in mice is embryonic lethal. One third die due to failure in chorioallantoic fusion and the rest due to placental vascular insufficiency and compromised vessel integrity (Mo et al., 2002). *Cyr61* is thus essential for vascular development in the placenta (Mo et al., 2002) and for growth, differentiation

and morphogenesis of the cartilage of the developing embryo (O'Brien and Lau, 1992). Expression data indicates *Cyr61* is a good target of Elf5, it was expressed at the right time and place to be an Elf5 target and it was differentially expressed upon *Elf5* knockdown. *Cyr61* was significantly down regulated at 24 and 48 hr after *Elf5* knockdown and it was also down regulated with differentiation. Down regulation with differentiation agrees with the fact that *Cyr61* is stimulated by FgF and TGF-β. All this data suggests *Cyr61* is an important target of Elf5 that is involved in the development of the placenta.

Sox2 is required in both the epiblast and trophectoderm lineages of the early embryo. It is also expressed in embryonic stem and trophoblast stem cells at high levels. The loss of *Sox2* causes the loss of pluripotency (Avilion et al., 2003). So the finding that *Sox2* was down regulated upon *Elf5* knockdown and growth factor removal, was important. Sox2 is expressed in the ICM of the blastocyst and later the anterior neuroectoderm of the embryo. In the extraembryonic tissues expression is initiated in the ExE and is maintained in the ExE part of the chorion (Avilion et al., 2003). Our *in situ* data confirmed this with mRNA expression in the ExE at D6.5. *Sox2* homozygous mutants die a short time after implantation, this is due to failure to maintain epiblast which also leads to problems in the extraembryonic tissues (Avilion et al., 2003). A rescue experiment with wild type ES cells (ES cells only contribute to the epiblast of the embryo) and *Sox2* homozygous mutant blastocysts allowed survival beyond implantation, but embryos died around E7.5-8.5 due to failure to develop the chorion (Avilion et al., 2003). This proved that Sox2 is vital for the extraembryonic tissues as death was not due to failure of the epiblast. Accordingly TS cells could not be isolated from *Sox2* mutant embryos as the tissue that they originate from was missing. This data indicates *Sox2* is essential for the developing embryo, placenta and TS cell maintenance and proliferation.

Wdr40B is part of the WD repeat protein family and has an unknown function. This family is thought to be involved in a variety of processes including the cell cycle, signal transduction, apoptosis and gene regulation (Lawson and Zhang, 2009). *Wdr40B* was expressed in the ExE and was down regulated when *Elf5* was

knocked down or differentiation was induced. Although it has an unknown function it could have an important role in any of the above processes.

Mme encodes the enzyme neprilysin, a membrane metallo-endopeptidase that is involved in tachykinin metabolism in the mammalian uterus (Patak et al., 2005). Tachykinins cause contraction in the uterine smooth muscle of rats, mice and humans (Patak et al., 2005). *Mme* was expressed in the ExE and EPC at D6.5, although it was expressed in the same place as *Elf5*, it does not appear to be a direct Elf5 target. Expression is only changed 48 hr after *Elf5* knockdown and not at a great level.

According to the whole mount *in situ* hybridisation data gained here, *Calca* appears to be expressed in the node. The node is the organiser of the embryo. The node specifies the fates of the different cell populations and then organises the different tissues in a body plan along the neural tube (Davidson and Tam, 2000). This was not expected as *Calca* was rapidly down regulated upon *Elf5* knockdown and more so upon growth factor removal. Expression was expected to be in the ExE. *Calca* levels in mouse trophoblast stem cells were quite low, between 0.01-0.02 (relative to the geomean) whereas genes like *Elf5, Sox2* and *Cyr61* were expressed at levels of 0.2-0.5 (relative to the geomean). If TS cells were the only site of *Calca* expression it would probably be picked up in the ExE. But because it was also expressed in the node at high levels, if the probe is left to develop any longer the dye would have just spread over the entire embryo leading to non specific staining.

Ly6a, lymphocyte antigen 6 complex (Sca1) is a marker for hematopoietic stem cells (Ottersbach and Dzierzak, 2005). *Ly6a* is therefore expressed in all known hematopoietic tissues. *Ly6a* was expressed in the extra embryonic tissues during the development of the placenta. It was expressed in the extra embryonic ectoderm and at lower levels in the ectoplacental cone at E6 -7.5 of development. Later on in development it is also expressed in foetal vessels of the labyrinth, the umbilical artery and the major vessels of the yolk sac (Ottersbach and Dzierzak, 2005). After 24 hr of knockdown *Ly6a* was up-regulated, this

confirms the microarray results. Unfortunately there was also a strong si negative effect in the 24 hr samples. The change in expression was still significant compared to the si negative control. This gene was interesting as it was one of the few genes that did not follow the same trend upon *Elf5* knockdown as growth factor removal (it is down regulated significantly upon growth factor removal).

Secretin is a gene characteristic of the placenta and is expressed in TS cells (Tanaka et al., 2002). *Secretin* was expressed in the EPC and was up regulated upon growth factor removal and the knockdown of *Elf5*. This gene is possibly involved in TS cell differentiation into other cell types.

Deletion of the Drosophila homolog of *Elavl2* (embryonic lethal abnormal vision like 2), as its name suggests, is embryonic lethal due to the abnormal development of neurons (Robinow et al., 1988). *Elavl2* has been found to be expressed in the developing mouse oocyte and the surrounding granulosa cells at all stages of folliculogenesis (Malcuit et al., 2009). *Elavl2* expression declines after the first cleavage stage (Malcuit et al., 2009). In this study *Elavl2* expression was found at very low levels in the ExE at E6.5 and 7.5 in mouse embryos. This corresponded to very low expression in TS cells. *Elavl2* was down regulated when TS cells differentiated. However, *Elavl2* did not appear to be an Elf5 target as it underwent the same changes in expression as the si negative control.

Spi8 (serine protease inhibitor 8), belongs to the Serpin super family of protease inhibitors that bind irreversibly to target proteases. Its physiological role is unknown but *in vitro* experiments suggest that it may inhibit Furin by means of analysis of binding sites and interactions (Gillard et al., 2006). In the mouse *Spi8* is highly expressed in the kidney with lower levels in the brain, liver, lymph nodes and the skin (Gillard et al., 2006). *Furin* is expressed in the ExE during early development and is required to activate Nodal in the epiblast which is required for anterio-posterior patterning. Furin is also involved in the activation of Bmp4 (Beck et al., 2002). Therefore Spi8 may play a role in regulating Furin during this stage of development as it too is expressed in the ExE at the same time as Furin.

When *Elf5* was knocked down or the TS cells differentiated *Spi8* was down regulated. Elf5 could thus play an indirect role in embryonic patterning.

Hmga2 is a member of the high mobility group A family that encodes a small chromatin associated protein that can alter transcription by changing chromatin structure. Hmga2 is expressed throughout foetal development and is associated with proliferative undifferentiated cells and tissue. As development continues expression becomes more restricted (Hirning-Folz et al., 1998). *Hmga2* encodes a stem cell factor that controls the maintenance and self renewal of neural stem cells in young mice. In *Hmga2* knockout mice it was shown that it is not required for the generation of neural stem cells but it is required for proliferation and self renewal (Nishino et al., 2008). The knockout of *Hmga2* also results in a pygmy phenotype with a very low body weight (Zhou et al., 1995). There is no published data relating to *Hmga2* in the placenta, but it could play a similar role in TS cells. *Hmga2* is down regulated upon the knockdown of *Elf5* and growth factor removal.

Zic3 is a zinc finger transcription factor, Zic family members are expressed in multiple tissues during development. *Zic3* is required for left-right asymmetry as mutations in *Zic3* cause X-linked heterotaxy in humans which results in abnormal positioning of internal organs with respect to each other in the L-R axis (Purandare et al., 2002). In mice a knockout of *Zic3* causes embryonic lethality in 50 % of mice, and another 30 % die postnataly. These mice have defects in turning, cardiac development, neural tube closure, and vertebral and rib defects (Purandare et al., 2002). *Zic3* is expressed in the ExE at day 5.0 and in some epiblast cells. As gastrulation begins, expression in the ExE declines and *Zic3* begins to be expressed in the tissue that is lined by mesoderm that emerges from the primitive streak to encircle the embryonic portion of the egg cylinder. It then goes on to be expressed in the anterior definitive endoderm. *Zic3* is expressed in the ectoderm adjacent to the node but not in the node (Elms et al., 2004), our *in situ* data has confirmed these findings. Zic3 induces the formation of the neural crest (Kitaguchi et al., 2000). Zic3 has been shown to act upstream of Nodal (Purandare et al., 2002) and Pitx2 (Kitaguchi et al., 2000) in L-R

asymmetry as their expression is random in *Zic3* mutants. Zic3 is thus essential for L-R specification and also anterior-posterior axis patterning (Elms et al., 2004; Kitaguchi et al., 2000; Klootwijk et al., 2000; Purandare et al., 2002). Although *Zic3* was not expressed in the ExE at the time points studied, it is expressed in the ExE at day 5.0 (Elms et al., 2004). *Zic3* is likely to be an indirect target of *Elf5* as it was slow to change expression upon *Elf5* knockdown and it is not expressed in the ExE.

Keratin7 is a cytosketetal protein. Keratin 7 is expressed in all subtypes types of trophoblast in a full term placenta (Pötgens et al., 2001). It was expressed at high levels in the EPC at E6.5-7.5, it was not expressed in the ExE. Keratin 7 was up regulated upon *Elf5* knockdown and differentiation. Elf5 could act to prevent the expression of this gene in undifferentiated TS cells.

In the embryo*, Bex1* (Brain-expressed X linked gene) is initially transcribed at the 2-cell stage of development and reaches a peak at the 4 to 8 cell stage of development. Expression then declines at the morula stage and rapidly increases at the expanded blastocyst stage (Williams et al., 2002). The expression of *Bex1* in isolated inner cell mass is 30 fold lower than that in the whole blastocyst indicating trophectoderm specific expression (Williams et al., 2002). *Bex1* is also expressed in ES cells (Hosler et al., 1989). In this study it was expressed in the ExE and EPC but it did not appear to be a target of *Elf5* as there was no change in gene expression upon knockdown. There may not have been a change in gene expression upon *Elf5* knockdown as *Bex1* expression levels are very high in TS cells, as high as the ubiquitous housekeeping genes.

Hopx was only expressed in the giant cells surrounding the embryo. Its expression data did not correlate with the microarray as it was up-regulated when initially studied. 1700112c13 RIKEN cDNA, is a peptidase (ingenuity). There is no published expression data on this gene, it was possibly expressed in the chorion in later embryos but the probe did not work well. It was dramatically down regulated upon *Elf5* knockdown and growth factor removal so it is a gene of interest but its role is unknown. GM784 is a fibronectin type 3 repeat, there is

no published knock out or expression data on this gene. It had mosaic expression in the ExE and seemed to be a good target.

The majority of these results correlated with the microarray with the 48 hr results being more exaggerated. These results are beginning to shed light on the role of Elf5 in the mouse trophoblast. *Elf5* seems to play a role in both the maintenance of TS cells and indirectly in embryonic patterning.

7.1.2 The over expression of Elf5

Progress has been made in the generation of an Elf5 inducible TS cell line. A 4-hydroxytamoxifen (OHT) inducible system was used where the Elf5-ER protein would not translocate into the nucleus and fulfil its transcriptional functions until OHT had been added to the culture. The VP16 transcriptional activation domain was inserted successfully to turn Elf5 into a potent activator and stable lines were generated. First by selection with Zeocin which was encoded on the plasmid, and secondly by selection with puromycin as time was limited. These lines had the plasmid integrated into their genome and expressed the protein. Immunocytochemical analysis showed that only a small amount of protein was going into the nucleus upon OHT addition. This was a problem as the concentration of OHT cannot be increased without affecting the expression of TS genes. This was due to the interaction of OHT with orphan estrogen related receptors expressed in TS cells.

The TS cell marker, the orphan estrogen related receptor Errβ, is structurally related to ERα and ERβ. Orphan nuclear receptors lack identified synthetic or natural ligands (Tremblay et al., 2001). Estrogen related receptors do not interact with natural estrogens, but OHT has been illustrated to disrupt Errβ binding with its co regulator protein and inhibit its transcriptional activity (Tremblay et al., 2001). This was a problem as Errβ is a stem cell marker required for trophoblast maintenance. The expression of stem cell markers will need to be monitored closely in future control experiments. The addition of 1 μg/ml of OHT did not affect the expression of the stem cell genes.

The gene expression of a few genes were analysed in preliminary experiments. Line 2 showed an increase in *Elf5* expression in comparison to the control. The changes in gene expression upon addition of 4-hydroxytamoxifen were not as straight forward as expected. *Sox2* for example looked like a very good target from the knockdown and expression data. But when Elf5 was turned into an activator it was down regulated instead of up regulated as expected. Another interesting fact was that the basal levels of genes were changed in the transfected cell lines compared to the control. This could have possibly been due to leaky expression from the plasmid. The up-regulation of Elf5 would have been interesting but clearly it is a complicated story. There have been further lines generated that are awaiting analysis but they unfortunately fall outside the scope of this thesis.

7.1.3 Changes to the TS cellular characteristics

The changes in morphology, DNA content, proliferation and apoptosis were investigated upon *Elf5* knockdown. These characteristics were investigated 5-6 days upon *Elf5* knockdown as no changes in morphology were seen 48 hr after the initial knockdown and because giant cells do not begin to appear until 4 days after the removal of growth factors (Erlebacher et al., 2004).

The knockdown of *Elf5* did have an effect on the morphology of the TS cells. The cells were larger compared to the untreated and si negative control. They did not look like the growth factor removal treatment but there was a definitive increase in the size of the nucleus and cytoplasm which is characteristic of giant cells. These morphological results were confirmed by DNA content analysis. There was an increase in the number of cells with a DNA content of 8N or greater which corresponds to endoreplicating cells. This increase in DNA content was not as great as the increase observed when growth factors were removed. There was a decrease in proliferation observed when the growth factors were removed, from 5 % to 1 % of cells in the late G2/M phase. There was no change in proliferation observed upon the knockdown of *Elf5*. There was also an increase in apoptosis

observed 5 days after the removal of growth factors, but that was not observed when *Elf5* was knocked down. The knockdown of *Elf5* did have an impact on the stem cellular characteristics of the TS cells. It was not as severe as the removal of growth factors as proliferation and apoptosis were not affected. Only a proportion of the cells displayed characteristics of giant cells. This could have been to differing levels of *Elf5* knockdown in each cell. The cells that did not receive the siRNA would have diluted out the effect of the siRNA.

Elf5 is part of the ETS family of transcription factors which are involved in the control of cell proliferation and tumorigenesis (Zhou et al., 1998). In the mouse mammary gland Elf5 is required for proliferation and differentiation of the alveolar epithelial cells during pregnancy and lactation (Zhou et al., 2005). A study by Oakes et al., (2008) found that Elf5 is required for the differentiation of $CD61^{+}$ luminal progenitor cells into the secretory alveolar lineage during pregnancy. Elf5 thus promotes differentiation in the mouse mammary gland. Elf5 seems to play a slightly different role in the mouse trophoblast, it acts to maintain the TS cells in an undifferentiated stem cell fate. By doing so it could act to preserve the ExE and prevent the precocious differentiation into the EPC/giant cells. It could also maintain cellular proliferation at an earlier time point, before the cells differentiate upon the loss of *Elf5*. These findings could explain why the ExE was missing in *Elf5* homozygous mutants. TS cells which reside in the ExE could not be maintained. Elf5 is a marker of undifferentiated TS cells and when *Elf5* was knocked out or down regulated, the TS cells differentiated into EPC precursors and finally giant cells (Donnison et al., 2005). This was further supported by the fact that Elf5 was down regulated upon differentiation. Furthermore most of the genes that were identified as Elf5 targets were affected the same way by *Elf5* knockdown and growth factor removal. This lends further support to the idea that Elf5 maintains the TS cells in an undifferentiated state.

7.1.4 Future work

The over expression of Elf5 will be a valuable tool to gain an understanding of the transcriptional networks Elf5 is involved in. Preliminary results have

demonstrated that the story is more complicated than once thought. There are other lines that need analysing for Elf5 over-expression by real time PCR, as they have been shown to have to plasmid inserted into their genome by PCR. These lines have been stored in liquid nitrogen for future use. It would also be interesting to over express Elf5 in ES cells as Ng and colleagues (2008) have done. They find that the over expression of Elf5 in ES cells causes differentiation into trophoblast cells. It would be interesting to see what genes are up-regulated when this occurs.

We investigated proliferation 6 days after the knockdown of *Elf5*. I think it would be valuable to examine proliferation at an earlier time point. Possibly 2-3 days after *Elf5* knockdown while *Elf5* levels are still low. I think there would be a decrease in cell proliferation upon knockdown. This is due to the fact that a few of the genes that Elf5 regulates are involved with proliferation and self-renewal.

The role of some of the target genes *in vivo* or *in vitro* could be elucidated by creating a knockout mouse or knockdown in TS cells. This way the Elf5 transcriptional networks involved in placental development may be revealed.

7.1.5 Conclusions

Elf5 is an essential transcription factor that is directly involved in the maintenance of the extraembryonic ectoderm and trophoblast stem cells. It may also be indirectly involved in embryonic patterning by the regulation of *Spi8* and *Zic3*. We have shown that Elf5 acts to maintain the trophoblast stem cells in an undifferentiated state. Elf5 may also play a role in TS cell proliferation but this has not been confirmed. The over-expression of Elf5 in the future will further verify its role in the placenta.

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9 Appendices

9.1 Materials

Mammalian cell culture reagents

Cell culture media

All reagents were kept at 4 °C. The protocols for cell culture media were obtained from Placenta and Trophoblast methods and protocols (Quinn et al., 2006).

2 x RPMI media

1 packet of RPMI Medium 1640 powder (Gibco, Invitrogen life technologies, Auckland, NZ)
500 ml MQ water
2.0 g Sodium Bicarbonate (Life Sciences, Sigma-Aldrich, USA)
HCl 10 M to adjust pH
Filter sterilised through a 0.22 µm filter

2 x DMEM F12

1 packet of DMEM F12 medium powder (Gibco, Invitrogen life technologies)
500 ml MQ water
2.438 g Sodium Bicarbonate (Life Sciences, Sigma-Aldrich, USA)
HCl 10 M to adjust pH
Filter steralised through a 0.22 µm filter

Mouse trophoblast stem cell media (mTS)

To make 200 ml:
100 ml of 2 x RPMI
40 ml Foetal calf serum (20 % FCS) (ICP Biologicals, Auckland, NZ)
2 ml of Sodium Pyruvate (1 mM) (100x Gibco, Invitrogen life technologies)
2 ml of Glutamax (2mM) (100x Gibco, Invitrogen life technologies)
364 µl β mercaptoethanol (0.1 mM) (Sigma-Aldrich, USA)
MQ water to 200 ml

Mouse embryonic fibroblast media (MEF media)

To make 200 ml:
100 ml of 2x DMEM F12
20 ml FCS (10%) (ICP biologicals, Auckland, NZ)
2 ml Sodium Pyruvate (1 mM) (100x Gibco, Invitrogen life technologies)
1 x Antibiotic/ Antimycotic (100x, 10,000 units of penicillin, 10,000 µg of streptomycin, 25 µg amphotercin B per ml) (Invitrogen life technologies)
MQ water to 200 ml

70 % Conditioned Media

70 % embryonic fibroblast conditioned media (see methods)
30 % mTS media
25 ng/ml Fgf4 (Sigma-Aldrich, USA)
1 µg/ml Heparin (Sigma-Aldrich, USA)

2x mTS freezing media
50 % FCS
30 % mTS media
20 % Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, UK)
Cool to 4 °C
Add 500 µl to 500 µl of cells in mTS media
Nalgene™ Cryo 1°C Freezing Container

PBS (DulbeccoA, OXOID limited, Basingstoke, Hampshire, England)
10 tablets were dissolved in 1L of MQ and then autoclaved

Growth Factors and Antibiotics
Fibroblast growth factor 4 human recombinant, 25 µg (Sigma-Aldrich, USA)
Resuspend in 1 ml PBS (Oxoid, UK) containing 0.1 % BSA (Invitrogen life technologies) to give a concentration of 25 µg/ml. Aliquot out into 50 µl aliquots and store at -80 °C. Dilute 1:1000 when adding to cells to give 25 ng/ml.

Heparin 10,000 units (Sigma-Aldrich, USA)
Dilute in PBS to give a 1 mg/ml stock, aliquot and store at -80°C. When needed thaw and keep at 4 °C. Add to media to a final concentration of 1 µg/ml.

Mitomycin C 2mg from *Streptomyces caespitosus* (Sigma-Aldrich, USA)
Resuspend in 2ml of PBS, aliquot and store at -80 °C.

Zeocin (invitrogen)
Puromycin (Sigma-Aldrich, USA)

Cell culture consumables
6 well plates (Nunc, Kamstrupvej, Denmark)
12 well plates (Nunc, Kamstrupvej, Denmark)
100 mm tissue culture dish (Nunc, Kamstrupvej, Denmark)
35 mm petri dishes (Falcon, BD, USA)
TrypLE™ Express (Gibco, Invitrogen life technologies Auckland, NZ)
Cryovials (Nunc, Kamstrupvej, Denmark)
Chamber slides- 2, 4 and 8 well (LAB-TEK, Nalgene Nunc International, USA)
Pipette Serological – 5 and 10 ml (LabServ, BioLab)
Pipette Serological -25 ml (Corning Incorporated, USA)
T75 tissue culture vented easy flask (Nunc, Kamstrupvej, Denmark)
T175 cm^2 Tissue culture vented cap (Nunc, Kamstrupvej, Denmark)

Transfection Reagents
OptiMEM reduced serum media (Gibco, Invitrogen life technologies)
Lipofectamine™ 2000 stored at 4 °C (Invitrogen life technologies, Auckland, NZ)
Reduced serum mTS media
Made up the same as mTS media but with only 5 % FCS instead

RNAi and DNA constructs
RNAi molecules are resuspended in DEPC water and stored at -80°C

Stealth Elf5 siRNA (Invitrogen life technologies) diluted to a working concentration of 50nM
Stealth siRNA negative control (Invitrogen life technologies) also diluted to a working concentration of 50nM
Elf5 mo ER pyCAGiZeo stored at -20°C (donated by Craig Smith, AgResearch, Ruakura), once modified also kept at -20°C.
P-Tet on Advanced plasmid (Clontech Lab Inc, USA)
Puromycin resistance plasmid (provided by Marion Woodcock, Transgenics group, AgResearch, Ruakura)

Molecular Biology Reagents

RNA Isolation and cDNA Synthesis

TRIZOL Reagent (Invitrogen life technologies)
pENSpike – provided by Craig Smith
MS2 RNA (Roche, Germany)
Linear Acrylamide (Ambion)
DNase buffer (Invitrogen life technologies)
DNase (Invitrogen life technologies)
25 mM EDTA (Invitrogen life technologies)
3M sodium acetate (BDH, England)
DEPC water and adjusted to pH 5.0 with 3M acetic acid
10 mM dNTP mix (Roche, Germany)
Oligo $dT_{14}VN$ (Invitrogen life technologies)
5 x First strand buffer (Invitrogen life technologies)
RNAseOUT™ Rnase Inhibitor (Invitrogen life technologies)
Superscript™ III Reverse Transcriptase (Invitrogen life technologies)
RNAse H™ (Invitrogen life technologies)

Real time PCR Reagents

2 x SYBR green master mix (Takara), kept at 4°C once thawed
MQ water
Primers (Sigma or Invitrogen)
Resuspend to 1 nmol/µl (1mM) in TE and make working stocks at 10pmol/µl or (10µM) in DEPC or MQ water.
Corbett Research gene discs
DEPC treated water
% diethyl pyrocarbonate (DEPC, Sigma-Aldrich, USA)
MQ water
Left mixing over night at room temperature
Autoclaved

Traditional PCR reagents

10 x PCR buffer with $MgCl_2$ (Invitrogen life technologies)
10mM dNTP Mix (Roche, Germany)
Fast start Taq Polymerase (Invitrogen life technologies)

Agarose (Invitrogen life technologies or Raylab, New Zealand)
Ethidium Bromide 10 mg/ml (Sigma-Aldrich)
Sybr® safe DNA gel stain- 10,000 x conc in DMSO (invitrogen, Molecular Probes)

Ampicillin stock 100 mg/ml (Sigma-Aldrich)

LB Broth
25g Luria Broth powder (Invitrogen life technologies)
Dissolve in 1L of MQ water
Autoclave and when cool add ampicillin to a final concentration of 100 µg/ml.
Store at 4 °C

LB/ampicillin plates
25g Luria Broth powder (Invitrogen life technologies)
1.5 g agar Difco™ Agar, Granulated, (BD, USA)
Dissolve in 1L of MQ water
Autoclave and when cool (about 50 °C) add ampicillin to a final concentration of 100 µg/ml. Pour into petri dishes and when set store at 4 °C with the agar up.

Ladders
100 bp DNA ladder (ABgene, Surrey, UK)
1 KB DNA ladder (ABgene, Surrey, UK)
Ultra ranger DNA ladder (ABgene, Surrey, UK)
Lambda DNA ladder , (Provided by Peter Pfeffer)
Gel loading buffer –6x loading buffer blue and red

DNA purification kits
GENECLEAN® II Kit (QBiogene)
Wizard gel clean up (Promega, Madison, USA)
Mermaid® Spin Kit (BIO 101, CA)
DNA purification maxi kit (QIAGEN Incorporated, Biolab scientific Limited, NZ)
Ligation mighty mix (Takara)

Invitrogen DH5α Max efficiency cells (>10^9/µg)
Thaw on ice and re-freeze and aliquots in a slurry of dry ice and isopropanol

Reagents for blue/white selection
40 µl of 20 mg/ml IPTG plus 20 µl of 20 mg/ml X-Gal spread onto an LB-Amp plate

Restriction enzymes
All from Roche, stored at -20°C EcoR1, Cla1, Pst1, Sac1, Sca1, Asp718, Nco1, Sph1, Sal1, Hpa1, BamH1, Spe1, Nhe1, Not1, Apa1, SacII/Ksp1, Xhol, HindIII, EcoRV, Ssp1 and Nae1.
Restriction buffers H, M, A, L, B (Roche, Indianapolis, USA)
Calf Intestinal phosphatase (Roche, Indianapolis, USA)
Buffers

Tris EDTA buffer
10mM Tris- HCl pH 8.0 (BDH, England)
1 mM EDTA
DEPC water
Autoclaved

T0.1 E Buffer
10mM Tris- HCl pH 8.0
0.1 mM EDTA
DEPC water
Autoclaved

50 x TAE buffer
2M Tris pH 8.0
0.57 % acetic acid (J.T Baker, USA)
500 mM EDTA
MQ water
Autoclaved

TBS buffer
25 mM Tris dissolved in MQ and adjusted to pH 7.5 with conc HCl
150 mM NaCl

TBS-tween
TBS buffer above with 0.1 % tween

Miniprep buffers
Alkaline Lysis Solution one
50 mM glucose
25 mM Trsi-Cl pH 8.0
10 mM EDTA pH 8.0
Autoclaved

Alkaline lysis Solution two
0.2 M NaOH
1 % SDS

Alkaline lysis Solution three
3 M potassium acetate
5 M glacial acetic acid
MQ water

Incubation Buffer (Proliferation)
0.5g Bovine Serum Albumin (Sigma) dissolved in 100 ml 1 x PBS and stored at 4 °C

Lysis buffer for genomic DNA isolation (store at RT)
100 mM NaCl
10 mM Tris pH 8.0
25 nM EDTA
0.5 % SDS
Proteinase K 1 mg/ml (Roche, Germany), added fresh before use.

Solvents

Phenol/chloroform (invitrogen life technologies, NZ)
Chloroform (AnalaR)
Isopropanol (LabServ, BioLab, Australia)
Ethanol (LabServ, BioLab, Australia)
Methanol (LabServ, BioLab, Australia)
Whole mount *in situ* Hybridisation Reagents
Paraformaldehyde 4 % in PBS (Sigma-Aldrich)
Proteinase K 25 mg (Sigma-Aldrich)
Glutaraldehyde 25 % (Sigma-Aldrich)
Yeast RNA 20 mg/ml (Sigma-Aldrich)
Heparin 50 mg/ml (Sigma-Aldrich)
Tween-20 (Polyoxyethylenesorbitan Monolaurate) 10 % and 0.1 % (Sigma-Aldrich)
RNase A (Sigma-Aldrich)
RNase Away (Molecular BioProducts Inc, San Diego, CA)
Maleic acid (Sigma-Aldrich)
Formamide (Fluka)
Quick spin columns for RNA (Boehringer Ingelheim, Germany)
Boehringer Blocking reagent (Boehringer Ingelheim, Germany)
BCIP (Boehringer Ingelheim, Germany)
NBT (Boehringer Ingelheim, Germany)
Anti-Dig Alkaline phosphatase (Boehringer Ingelheim, Germany)
Dig RNA labeling kit (Boehringer Ingelheim, Germany)
T7 RNA polymerase (Roche, Germany)
SP6 RNA polymerase (Roche, Germany)
Lamb serum (Gibco, invitrogen life sciences)
PBT- PBS plus 1 % Tween-20 added fresh
Glycerol 100 %
Lithium Chloride 4M

<u>1x MAB</u>
100 mM Maleic acid, dissolve and pH to 7.5
150 mM NaCl

<u>1x MABT</u>
100 mM Maleic acid, dissolve and pH to 7.5
150 mM NaCl
% Tween-20
MQ water

1x $MABT_{500}$
100 mM Maleic acid
500 mM NaCl
% Tween-20

<u>NTMT</u>
mM Tris (pH 9.5)
100 mM NaCl
50 mM $MgCl_2$

1 % Tween-20

Hybridisation Mix
Formamide 50 %
SSC, pH 5.0 with citric acid 1.3x
EDTA pH 8.0, 5 mM
Yeast RNA, 50 µg/ml
Tween-20 0.2 %
CHAPS 0.5 %
Heparin 100 µg/ml
MQ to 50 ml
Flow Cytometry reagents
Sheath Fluid (PBS)
Dulbecco's Phosphate Buffered Saline (Gibco)
IsoFlow (Beckman Coulter Inc, Fullerton, CA)

DNA extraction buffer
$NaHPO_4$ 200 mM
Citric Acid 100 mM
Mix 96 ml of solution A with 4 ml of solution B. Check pH = 7.8, if necessary titrate to pH 7.8 with solution B.

PI staining solution
To 9 ml of sterile PBS add 500 µl PI (50 µg/ml) of 1 mg/ml stock, and add 500 µl RNase A (50 µg/ml) of 1 mg/ml stock. Make fresh each time and keep in dark.

DNA beads for calibration- CaliBRITE™3 beads (BD Biosciences)
DNA QC particles (Becton Dickinson)
Propidium Iodide (invitrogen)
RNase A (invitrogen)
APO-BrdU™ TUNEL Assay Kit (Molecular Probes, invitrogen)
Nocodazole (Sigma-Aldrich)
Make 1 mg/ml stocks in MQ and dilute to a working concentration before use.
Hoechst 33342 nuclear dye (invitrogen)
Phalliodin (Sigma-Aldrich)

Antibodies
Histone H3 serine 10 phospho primary antibody (Upstate)
Estrogen receptor ligand binding domain primary antibody (Abcam)
IgG-rabbit Alexa 488 secondary antibody (invitrogen)

9.2 Raw Data

Table 16: Raw real time data for the 24 hrsamples when Elf5 was knocked down in mouse trophoblast stem cells.

Replicate mean of three measurements divided by the house keeping genes

24 hr	*Elf5*	*Cyr61*	*Sox2*	*Wdr40B*	*Calca*	*Mme*	*Bex1*	*1700112c13*	*Elavl2*	*Keratin 7*	*Ly6a*	*Zic3*	*Hopx*	*Gm784*	*Spi8*	*Hmga2*	*Secretin*
337 Elf5 KD	0.0945	0.0664	0.0806	0.0365	0.0056	0.0040	1.3635	0.0346	0.0037	0.3245	0.0760	0.0209	0.0256	0.0291	0.0227	0.2165	0.4550
337 Elf5 KD	0.0955	0.0626	0.0977	0.0332	0.0046	0.0060	1.5329	0.0351	0.0039	0.3289	0.0760	0.0188	0.0309	0.0275	0.0167	0.2565	0.3479
337 Elf5 KD	0.0781	0.0695	0.0910	0.0321	0.0055	0.0047	1.5355	0.0459	0.0038	0.2716	0.0721	0.0202	0.0254	0.0301	0.0238	0.2129	0.2808
733 Elf5 KD	0.0701	0.0920	0.0774	0.0272	0.0047	0.0083	1.7313	0.0386	0.0034	0.3590	0.0677	0.0136	0.0335	0.0291	0.0272	0.1438	0.2816
733 Elf5 KD	0.0830	0.0829	0.0846	0.0229	0.0038	0.0063	1.6808	0.0350	0.0025	0.4621	0.0732	0.0112	0.0390	0.0209	0.0274	0.1177	0.3131
733 Elf5 KD	0.0599	0.0751	0.0742	0.0224	0.0041	0.0066	1.5972	0.0326	0.0032	0.3890	0.0671	0.0119	0.0365	0.0204	0.0243	0.1251	0.2543
Si Negative	0.4744	0.1562	0.1283	0.0439	0.0074	0.0092	1.6103	0.0477	0.0034	0.2755	0.0548	0.0207	0.0294	0.0332	0.0294	0.2959	0.2169
Si Negative	0.3597	0.1521	0.1120	0.0472	0.0074	0.0076	1.7119	0.0444	0.0034	0.2138	0.0551	0.0149	0.0330	0.0344	0.0291	0.2452	0.1935
No Treatment	0.4213	0.1895	0.1211	0.0438	0.0104	0.0107	1.5050	0.0449	0.0049	0.2297	0.0393	0.0186	0.0318	0.0345	0.0324	0.2270	0.2142
No Treatment	0.4366	0.1890	0.1255	0.0421	0.0102	0.0097	1.5610	0.0472	0.0038	0.2451	0.0398	0.0145	0.0276	0.0297	0.0336	0.2063	0.2095
No Growth Factors	0.1770	0.1273	0.0101	0.0092	0.0004	0.0101	1.7264	0.0009	0.0011	0.4368	0.0085	0.0008	0.0246	0.0086	0.0180	0.0787	0.2693
No Growth Factors	0.1481	0.1212	0.0065	0.0090	0.0004	0.0095	1.5024	0.0005	0.0010	0.4240	0.0080	0.0005	0.0189	0.0070	0.0139	0.0709	0.3138

Table 17: Raw real time data for the 48 hrsamples when Elf5 was knocked down in mouse trophoblast stem cells.

Replicate mean of three real time measurements divided by the mean of three house keeping genes

48 hr	*Elf5*	*Cyr61*	*Sox2*	*Wdr40B*	*Calca*	*Mme*	*Bex1*	*1700112c13*	*Elavl2*	*Keratin 7*	*Ly6a*	*Zic3*	*Hopx*	*Gm784*	*Spi8*	*Hmga2*	*Secretin*
337 Elf5 KD	0.0841	0.0156	0.0823	0.0171	0.0070	0.0056	0.4889	0.0333	0.0009	0.2033	0.0630	0.0070	0.0219	0.0029	0.0197	0.0505	0.1323
337 Elf5 KD	0.0822	0.0355	0.1470	0.0256	0.0095	0.0132	0.8835	0.0514	0.0023	0.2018	0.0839	0.0097	0.0360	0.0092	0.0311	0.0950	0.1062
337 Elf5 KD	0.0951	0.0333	0.1792	0.0273	0.0102	0.0126	1.0730	0.0561	0.0020	0.2284	0.0933	0.0081	0.0401	0.0099	0.0321	0.0926	0.1305
733 Elf5 KD	0.0488	0.0229	0.0669	0.0094	0.0036	0.0053	1.0749	0.0126	0.0009	0.2754	0.0290	0.0033	0.0142	0.0027	0.0094	0.0658	0.0885
733 Elf5 KD	0.0501	0.0490	0.1003	0.0174	0.0057	0.0109	1.6129	0.0138	0.0013	0.2233	0.0442	0.0052	0.0229	0.0065	0.0165	0.1049	0.0928
733 Elf5 KD	0.0561	0.0458	0.0664	0.0111	0.0033	0.0111	1.1619	0.0104	0.0013	0.1849	0.0319	0.0036	0.0190	0.0045	0.0117	0.0923	0.0696
Si Negative	0.5106	0.1010	0.2961	0.0565	0.0371	0.0200	1.3774	0.1624	0.0016	0.1766	0.0699	0.0092	0.0314	0.0201	0.0690	0.1173	0.0751
Si Negative	0.4645	0.1010	0.3204	0.0525	0.0274	0.0185	1.2500	0.1556	0.0017	0.1444	0.0710	0.0118	0.0280	0.0201	0.0676	0.1129	0.0688
No Treatment	0.4477	0.1469	0.3142	0.0469	0.0267	0.0188	1.0124	0.1416	0.0042	0.1157	0.0631	0.0114	0.0282	0.0198	0.0598	0.1568	0.0484
No Treatment	0.5087	0.1310	0.3201	0.0410	0.0251	0.0143	1.1181	0.1667	0.0034	0.1377	0.0665	0.0112	0.0290	0.0142	0.0589	0.1187	0.0568
No Growth Factors	0.1155	0.0348	0.0074	0.0142	0.0002	0.0026	1.2665	0.0004	0.0002	0.4136	0.0013	0.0053	0.0063	0.0015	0.0096	0.0630	0.3157
No Growth Factors	0.0714	0.0125	0.0044	0.0142	0.0004	0.0014	0.6563	0.0011	0.0005	0.4499	0.0012	0.0014	0.0026	0.0006	0.0126	0.0632	0.2751

Table 18: Raw real time data for the geometric mean of three house keeping genes for the knockdown of Elf5.

	copies per microlitre			
SAMPLE 24 hr	GAPDH	Bactin	Btubulin	Geomean
337 Elf5 KD	628808.1	89961.3	19764.66	103789.7
337 Elf5 KD	628808.1	68644.76	19764.66	94842.92
337 Elf5 KD	732118.1	71739.98	25196.06	109788.9
337 Elf5 KD	595691.5	85224.81	22120.04	103942
337 Elf5 KD	700666.8	131366.1	24756.12	131590.9
337 Elf5 KD	772819.1	144919.6	25196.06	141312
337 Elf5 KD	407891.4	52379.22	10057.95	59896.89
337 Elf5 KD	407891.4	52379.22	8986.958	57690.65
337 Elf5 KD	403754.6	57783.33	12120.05	65635.91
733 Elf5 KD	869958.1	124449.7	34703.45	155461
733 Elf5 KD	869958.1	146373.6	29310.8	155118.3
733 Elf5 KD	1012888	161475.4	35320.16	179430.7
733 Elf5 KD	506443.9	89961.3	23400.99	102158.3
733 Elf5 KD	534598.9	76486.84	22120.04	96709
733 Elf5 KD	558595.8	94017.7	25196.06	109785.9
733 Elf5 KD	869958.1	163095.6	32803.81	166963.5
733 Elf5 KD	700666.8	124449.7	34703.45	144641.4
733 Elf5 KD	861135	211619	37365.51	189539.2
Si Negative	869958.1	138667	34703.45	161168.9
Si Negative	918322.1	154508.5	36713.09	173349.6
Si Negative	909008.5	152973.7	44240.09	183231.5
Si Negative	628808.1	105809.5	24756.12	118097.8
Si Negative	628808.1	131366.1	22120.04	122253.8
Si Negative	772819.1	161475.4	25196.06	146500.5
No Treatment	1341133	251396.7	51464.87	258889.3
No Treatment	1341133	280116.6	48647.73	263405.3
No Treatment	1739916	262732.3	52379.45	288226.7
No Treatment	1494395	202488.7	45984.79	240525
No Treatment	1415691	172159.8	48647.73	228027.7
No Treatment	1561475	248899.4	52379.45	273048.6
MTS Control	1415691	295684.5	64462.06	299939.6
MTS Control	1494395	312117.6	64462.06	310953.8
MTS Control	1739916	363455.7	58621.59	333436
MTS Control	1665170	295684.5	60933.47	310728.5
MTS Control	1577473	312117.6	60933.47	310726.8
MTS Control	1836644	383655.3	73426.18	372625.3

	copies per microlitre			
SAMPLE 48 hr	GAPDH	Bactin	btubulin	Geomean
337 Elf5 KD	814700.1	149658.2	34482.89	161398.9
337 Elf5 KD	1006532	185720.3	38615.31	193263
337 Elf5 KD	1012538	184150.5	43854.09	201464.3
337 Elf5 KD	3978506	608882.8	76154.35	569267.2
337 Elf5 KD	3978506	517862.5	80588.42	549628.2
337 Elf5 KD	4944625	637216.2	86485.91	648318.6
337 Elf5 KD	3978506	440448.6	76154.35	511018.5
337 Elf5 KD	4422164	490653.3	80588.42	559191.7
337 Elf5 KD	5213036	572014.9	91521.53	648645.1
733 Elf5 KD	1243532	243253	34482.89	218494
733 Elf5 KD	1243532	230472.1	36490.65	218685
733 Elf5 KD	1545505	315915	41441.19	272494.4
733 Elf5 KD	2001123	270980.2	45760.77	291676.6
733 Elf5 KD	2224275	301868	45760.77	313208.1
733 Elf5 KD	2359009	315915	51968.95	338335.8
733 Elf5 KD	3054450	546580.6	71964.25	493440.3
733 Elf5 KD	3054450	576891.2	76154.35	511965.3
733 Elf5 KD	3415321	637216.2	81727.35	562378.3
Si Negative	2345017	301868	34482.89	290083
Si Negative	2472312	301868	36490.65	300862
Si Negative	3072675	315915	39161.04	336238.2
Si Negative	2224275	286007.5	30792.7	269569.8
Si Negative	2109751	286007.5	30792.7	264861.4
Si Negative	3072675	371440.7	37006.36	348252.4
No Treatment	3395063	395381	57385.88	425490.2
No Treatment	3579358	395381	60727.16	441300.7
No Treatment	4219501	436725.6	58196.89	475105.3
No Treatment	2109751	218362.8	29098.45	237552.6
No Treatment	2224275	230472.1	29098.45	246164.6
No Treatment	2622071	268689.7	34970.22	290981
MTS Control	3220256	490653.3	60727.16	457807.3
MTS Control	3395063	517862.5	64262.99	483438.3
MTS Control	4448551	603736	72981.29	580888
MTS Control	533750.8	127286.2	11118.47	91072.71
MTS Control	533750.8	108258.5	11118.47	86287.62
MTS Control	663363.9	140596.4	11932.12	103629

Table 19: Proliferation results for the knockdown of *Elf5* in mouse trophoblast stem cells. The average and the standard error of the mean was calculated.

No treatment	Si negative	337	733	No Gf	Nocodazole 15 ng	Noc 30ng	Noc 50 ng	Mito 1ug
5.14	3.92	4.42	4.82	1.27	6.52	8.87	14.37	2.5
5.47	4.41	5.25	5.29	1.02	6.18	8.06	14.1	2.67
5.3	3.9	4.62	4.98			8.51	14.85	2.67
5.12	4.31	5.13	5.83				14.13	
4.92		5.02	6.2				13.82	
3.88							13.66	
4.2								
3.83								
4.16								

Table 20: Apoptosis results for the knockdown of *Elf5* in mouse trophoblast stem cells. Duplicates were done for each time point.

	48 hr	48 hr	5 days	5 days
337	0.36	0.39	0.98	0.98
733	0.24	0.3	0.84	0.91
si negative	0.76	0.75	0.83	0.84
No treatment	0.4	0.48	0.4	0.39
No Growth factors	0.38	0.51	7.4	8.2

Table 21: DNA content results for the knockdown of *Elf5* in mouse trophoblast stem cells

	No Treatment				Ave	sem
2N	45.0	42.0	43.0	46.9	44.2	1.1
4N	43.0	46.0	44.0	43.7	44.2	0.6
>8N (M3)	8.8	9.0	9.0	6.2	8.3	0.7
>8N (M4)	2.2	2.5	2.8	2.7	2.6	0.1
Total (M3 + M4)	11.0	11.5	11.8	8.9	10.8	

	Si Negative				Ave	sem
2N	50.0	52.0	54.0	55.0	52.8	1.1
4N	40.0	40.0	39.0	38.5	39.4	0.4
>8N (M3)	8.5	8.0	7.1	6.3	7.5	0.5
>8N (M4)	1.7	1.7	1.3	1.1	1.5	0.1
Total	10.2	9.7	8.4	7.4	8.9	

337 Elf5 knockdown					Ave	sem
2N	48.0	51.0	49.0	50.0	49.5	0.6
4N	32.0	32.0	32.0	32.0	32.0	0.0
>8N (M3)	13.0	12.0	13.0	12.0	12.5	0.3
>8N (M4)	4.4	4.0	5.2	4.7	4.6	0.3
Total	17.4	16.0	18.2	16.7	17.1	

733 Elf5 knockdown					Ave	sem
2N	47.3	47.6	48.2	48.6	47.9	0.3
4N	31.9	32.6	32.3	32.5	32.3	0.2
>8N (M3)	14.1	14.1	13.4	13.8	13.8	0.2
>8N (M4)	6.0	5.0	5.0	4.7	5.2	0.3
Total	20.1	19.0	18.3	18.5	19.0	

No Growth factors					Ave	sem
2N	25.5	26.5	25.5	25.7	25.8	0.3
4N	37.2	35.3	35.2	35.9	35.9	0.7
>8N (M3)	22.8	23.5	24.0	23.3	23.4	0.4
>8N (M4)	11.4	11.0	11.9	11.3	11.4	0.3
Total	34.2	34.5	35.9	34.6	34.9	

Table 22: Raw real time data for the geometric mean of the three housekeeping genes for Elf5 overexpression.

SAMPLE	GAPDH	Bactin	Btubulin	Geomean
Control 0 Tx	1.90E+06	3.89E+05	1.50E+05	480336.6
Control 0 Tx	2.00E+06	5.34E+05	1.50E+05	543389.6
Control 0 Tx	2.12E+06	5.66E+05	1.77E+05	596987.5
Control 0.5 ug/ml	2.00E+06	2.98E+05	1.09E+05	402738.6
Control 0.5 ug/ml	1.90E+06	3.15E+05	1.15E+05	409897.8
Control 0.5 ug/ml	1.72E+06	3.71E+05	1.29E+05	434735.4
Control 1ug/ml	1.38E+06	2.29E+05	8.39E+04	298493.8
Control 1ug/ml	1.62E+06	2.29E+05	8.85E+04	320290.6
Control 1ug/ml	1.63E+06	2.56E+05	9.90E+04	345737.1
Line 1 0 Tx	1.01E+06	6.11E+04	6.44E+04	158290.5
Line 1 0 Tx	9.06E+05	7.96E+04	6.79E+04	169849.3
Line 1 0 Tx	1.07E+06	8.01E+04	8.01E+04	189919.6
Line 1 0.5 ug/ml	1.90E+06	2.69E+05	1.28E+05	402738.6
Line 1 0.5 ug/ml	1.80E+06	2.55E+05	1.28E+05	388793.3
Line 1 0.5 ug/ml	2.12E+06	2.70E+05	1.59E+05	450328.5
Line 1 1ug/ml	1.46E+06	1.76E+05	1.04E+05	298493.8
Line 1 1ug/ml	1.38E+06	1.85E+05	1.04E+05	298493.8
Line 1 1ug/ml	1.63E+06	2.07E+05	1.22E+05	345737.1
Line 2 0 Tx	1.18E+06	1.67E+05	1.15E+05	283125.2
Line 2 0 Tx	1.31E+06	1.76E+05	1.15E+05	298493.8
Line 2 0 Tx	1.25E+06	1.97E+05	1.43E+05	327936.1
Line 2 0.5 ug/ml	1.06E+06	2.42E+05	1.28E+05	320290.6
Line 2 0.5 ug/ml	9.55E+05	2.55E+05	1.28E+05	314696.6
Line 2 0.5 ug/ml	1.32E+06	3.17E+05	1.51E+05	398074
Line 2 1ug/ml	1.18E+06	2.83E+05	1.28E+05	349788.4
Line 2 1ug/ml	1.24E+06	2.69E+05	1.35E+05	356006.3
Line 2 1ug/ml	1.47E+06	3.34E+05	1.68E+05	434735.4

Table 23: Raw values for the over expression of Elf5. These values are shown as expression relative to the geometic mean of *Gapdh, βactin* and *βtubulin*.

	Elf5	*Sox2*	*Calca*	*Secretin*	*Wdr40B*
Control 0 Tx	0.3563	0.1526	0.0083	0.1425	0.0470
Control 0.5 ug/ml	0.2968	0.1431	0.0089	0.1177	0.0432
Control 1ug/ml	0.2570	0.1540	0.0098	0.1584	0.0427
Line 1 0 Tx	0.2654	0.2479	0.0089	0.1064	0.0382
Line 1 0.5 ug/ml	0.1596	0.1659	0.0124	0.0351	0.0228
Line 1 1ug/ml	0.1657	0.1465	0.0134	0.0367	0.0195
Line 2 0 Tx	0.5881	0.2106	0.0007	0.4545	0.0195
Line 2 0.5 ug/ml	0.6724	0.0529	0.0032	0.1142	0.0090
Line 2 1ug/ml	0.7584	0.0525	0.0024	0.1318	0.0088