The Development of Genetic Models to Understand the Role of the Wilms' Tumour Suppressor Gene, *WT1*

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"Discovery is seeing what everybody else has seen, and thinking what nobody else has thought."

Albert Szent-Gyorgi
(Nobel Prize in Physiology and Medicine 1937)

Or

"Do, or do not. There is no 'try'."

Yoda ('The Empire Strikes Back')

Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Abstract

Wilms' tumour is a paediatric kidney cancer that arises during embryogenesis from pluripotent renal precursor cells. Multiple genetic loci have been linked to the etiology of Wilms' tumour, but only the Wilms' tumour suppressor gene, *WT1*, has been cloned and shown to play a direct role in Wilms' tumourigenesis. In humans, germline mutations of *WT1* are associated with both Wilms' tumours and urogenital malformations. Analysis of the mouse knockout for WT1 revealed a failure of kidney and gonad development, establishing a fundamental role for WT1 in early urogenital development. The *WT1* gene encodes multiple isoforms of a nuclear protein containing four zinc fingers which acts as a transcription factor and is predicted to have a role in RNA processing. Although there are a number of clues as to the function of WT1, important questions remain unanswered.

The WT1 knockout is embryonic lethal, dying at midgestation. This prevents insight into the role of WT1 in later embryogenesis and post-natal development. Also the embryonic lethality of the WT1 knockout mouse precludes any studies of the role of WT1 in Wilms' tumourigenesis. The characterization of WT1 as a transcriptional regulator suggests that identification of its target genes may provide insight into its function at various stages of normal development and during tumorigenesis. Whilst numerous WT1 target genes have been suggested, mainly through the use of promoter-reporter constructs in cell lines *in vitro*, many lack evidence to indicate a physiological relevance. With the limitations of these previous studies in mind, this thesis describes the use of gene targeting in embryonic stem cells, to develop two different genetic systems to facilitate the investigation of WT1 function.

Firstly, using the *Cre/LoxP* system a conditional null allele for WT1 was generated by targeting the WT1 locus in embryonic stem cells. *LoxP* sites were positioned either side of exon 1 of *WT1*, and correctly targeted embryonic stem cells were used to generate mice. Together with tissue-specific transgenes directing expression inducible and non-inducible forms of *Cre* recombinase *in vivo*, this conditional WT1 mouse model will provide a powerful tool to further investigate the role of WT1 in development and disease.

Secondly, WT1 null embryonic stem cells were generated through sequential gene targeting of both alleles of *WT1*. These WT1 null embryonic stem cells were used to identify endogenous genes regulated, directly or indirectly, by WT1 and also to identify interacting protein partners. To identify candidate WT1 target genes, WT1 null embryonic stem cells were studied in an *in vitro* differentiation system. Analysis of gene expression profiles using Affymetrix Genechip arrays, identified a set of differentially expressed transcripts, which may represent WT1 regulated genes. Interestingly, these included Wnt-4 and BF-2, two genes crucial for the development of the kidney.

The biological activity of WT1 is modulated by its interaction with several known protein partners. To identify other protein partners, *in vitro* differentiation of embryonic stem cells, followed by immunoprecipitation of endogenous WT1 associated complexes coupled to MALDI-TOF analysis, was used to identify interacting protein partners. From this study, an interaction of WT1 with heterogeneous nuclear ribonucleoprotein U (hnRNP U) was identified, a protein involved in transcriptional repression, the packaging and processing of RNA, and chromatin remodeling.

Using a selection of lineage specific markers on differentiating embryonic stem cells, it was observed that WT1 is expressed in neuronal progenitors *in vitro*. Extending the analysis *in vivo*, WT1 was expressed in the ventral half of the neural tube in a domain that corresponds to location of neuronal progenitors. Specifically WT1 expression was detected in a progenitor domain that gives rise to motor neurons. WT1 expression was also present in motor neurons as shown by co-staining with Islet-1. The expression of WT1 in the progenitors of the neural tube opens the possibility that WT1 may have role in patterning of the neural tube.

In summary, the development of these two systems will provide important resources to further understand the function of WT1. The WT1 conditional null mouse will permit the role WT1 *in vivo* to be examined, whilst the WT1 null ES cells provide an *in vitro* experimental system in which to screen for candidate WT1 target genes and interacting protein partners. The power of this system has been demonstrated in this thesis by the identification of a set of candidate WT1 target genes and an interaction with hnRNP U.

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List of Abbreviations

°C	degrees centigrade
µg	micrograms
µl	microlitres
µM	micromolar
bp	base-pairs
BAG-3	Bcl-2 associated athanogene 3
BSA	bovine serum albumin
BF-2	Brain Factor 2
CTD	Carboxyl terminal domain
cDNA	complementary deoxyribonucleic acid
CTGF	Connective Tissue Growth Factor
Cre	Cre Recombinase
CRABP-II	Cellular retinoic acid binding protein II
dH_2O	distilled water
DDS	Denys-Drash Syndrome
DAPI	4',6-Diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DMEM	Dulbecco's MEM
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxyctosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
E	embryonic day
E14	E14.1 embryonic stem cell line
EC	Embryonal Carcinoma cell
EGR1	Epidermal Growth Receptor 1
IGFR	Insulin Like Growth Factor Receptor 1
E. coli	Escherichia coli
EDTA	ethylenediamine tetra-acetic acid
EST	expressed sequence tag
ES	Embryonic stem cell
Esg-1	Embryonic stem cell specific-1
EPDC	Epicardially Derived Cell
EBs	Embryoid Bodies
FAP	Familial Adenomatous Polyposis
FOG2	Friend of Gata-2
FS	Frasier Syndrome
G418	Geneticin
GANC	Gancyclovir
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
g	grams
GDNF	Glial cell line derived neurotrophic factor
HnRNP U	Heterogenous Nuclear Ribonucleoprotein U
HsvTK	herpes simplex virus thymidine kinase
HCL	Hydrochloric acid
HSP-70	Heat shock protein -70
IGFBP	Insulin Like Growth Factor Binding Protein
l	litres
ITSFn	Insulin, transfferin, selenium and fibronectin

IGF	Insulin Growth Factor
K1	WT1 null embryonic stem cell Line
kb	kilobase-pairs
LCR	locus control region
LIF	Leukemia Inhibitory Factor
MALDI-TOF	Matrix assisted laser desorption/ionization time of flight mass
M	molar
mg	milligrams
ml	millilitres
MOPS	Morpholinopropanesulfonic acid
mM	millimolar
mRNA	messanger ribonucleic acid
Nedd4	Neural precursor cell Expressed Developmentally Downregulated
Neo	Neomycin
PAGE	Polyacrylamide Gel Electrophoresis
par4	Prostrate Apoptosis Response Protein 4
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF-A	Platelet Derived Growth Factor A
PIN	prostatic intraepithelial neoplasia
PGK	phosphoglycerate kinase promoter
NOV	Neproblastomas overexpressed
IP	Immunoprecipitation
RA	*All-trans* Retinoic Acid
RAR-α	retinoic acid receptor α
RALDH2	retinaldehyde dehydrogenase II
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
rpm	revolutions per minute
RNA Pol II	RNA Polymerase II
RRM	RNA Recognition Motif
SAF-A	Scaffold Attachment Factor A
SDS	sodium dodecyl (lauryl) sulphate
SSC	saline sodium citrate
SRF	Serum Response Factor
TBST	Tris Buffered Saline and Tween
TAE	Tris-Acetate-EDTA
Tris	tris hydroxymethyl aminomethane
YAC	Yeast Artifical Chromosome
WAGR	Wilms' Tumour Anirida and Mental Retardation
WISP-1	Wnt-1 Induced Secreted Protein
WT1	Wilms' Tumour Suppressor 1
WTAP	Wilms' Tumour Associated Protein

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Chapter 1 Introduction

Chapter 1 Introduction

1. Wilms' Tumour and Mammalian Kidney Development

Wilms' tumour is a paediatric kidney malignancy of embryonic origin that has an incidence of 1 out of 10,000 infants (Breslow *et al.*, 1988). It serves as a striking example of cancer arising through inappropriate development (Hastie, 1994). To begin to understand the how Wilms' tumours develop, it is important to understand how normal mammalian kidney development proceeds (For reviews see Saxen, 1987; Lechner and Dressler, 1997; Kuure *et al.*, 2000).

In mammals, the kidney develops in three stages: pronephros, mesonephros and metanephros. Kidney development begins with the differentiation of the intermediate mesoderm to form the pronephric duct. The pronephric duct contributes to the formation of the first kidney, the pronephros. The pronephros is a transitory, non-functional structure which develops early during development, at around E8 in the mouse. In mammals, the pronephric duct degenerates, leaving only the caudal portion, which forms the Wolffian duct.

The next stage is the development of the mesonephros, which begins at around E9.5 in the mouse. Initiation of the mesonephros is triggered when the pronephric duct reaches the presumptive mesonephric mesenchyme and induces the mesenchymal cells to condense. Condensation of the mesenchyme leads to the formation of the mesonephric tubules that develop into nephrons. The mesonephric nephrons range from simple epithelium to convoluted tubules complete with glomeruli. The mesonephros remains the permanent kidney in amphibians and fish, and may also function transiently in mammals. Eventually, however the mammalian mesonephros starts to regress and this leads to complete absence of the organ in females by around E15. In males, the caudal portions of the tubules and Wolffian duct remain and develop into structures of the male genitals.

The third and final structure to develop is the functional mammalian kidney, the metanephros, which starts to form around E10.5-E11 in the mouse (Figure 1.1). Development of the metanephros is dependent upon reciprocal inductive interactions between the epithelial ureteric bud and metanephric mesenchyme. The ureteric bud develops from the caudal portion of the Wolffian duct, through inductive signals from the metanephric mesenchyme (Figure 1.1 (a)). During recent years, experiments have indicated that the Ret/GDNF signalling pathway is part of this inductive event which initiates ureteric bud outgrowth. GDNF is first expressed in the metanephric blastema before induction. Its cognate receptor, c-Ret, is expressed in the mesonephric duct and becomes restricted to the growing ureteric tip once induction occurs. The evidence to support the roles for GDNF and c-Ret in providing the inductive signal for ureteric bud initiation, comes from analysis of the mouse knockouts of either GDNF or c-Ret (Schuchardt *et al.*, 1994; Sanchez *et al.*, 1996; Pichel *et al.*, 1996). Both mouse models exhibit kidney agenesis or dygenesis, as a result of a failure of the ureteric bud to develop from the mesonephros.

Once initiated, the ureteric bud then invades the metanephric mesenchyme and branches (Figure 1.1 (b) and (c)). Upon contact with the ureteric bud, the metanephric mesenchyme is induced to condense along the surface of the bud and begins to form a blastema of cells adjacent to the tips of the branching ureteric bud. This marks the next stage in the development of the metanephric kidney in which the metanephric mesenchyme is induced to form nephrons (nephrogenesis). Signalling molecules expressed at the tips of the ureter initiate the induction of nephrogenesis. Studies using *in vitro* ureteric bud cell lines, and protein purification of conditioned medium from these cell lines, has identified leukaemia inhibiory factor (LIF) as being a signalling molecule that induces nephrogenesis (Barasch *et al*, 1999). Unlike the crucial role for GDNF and c-Ret, there appears to be some level of redundancy as the LIF knockout mouse does not show a kidney phenotype

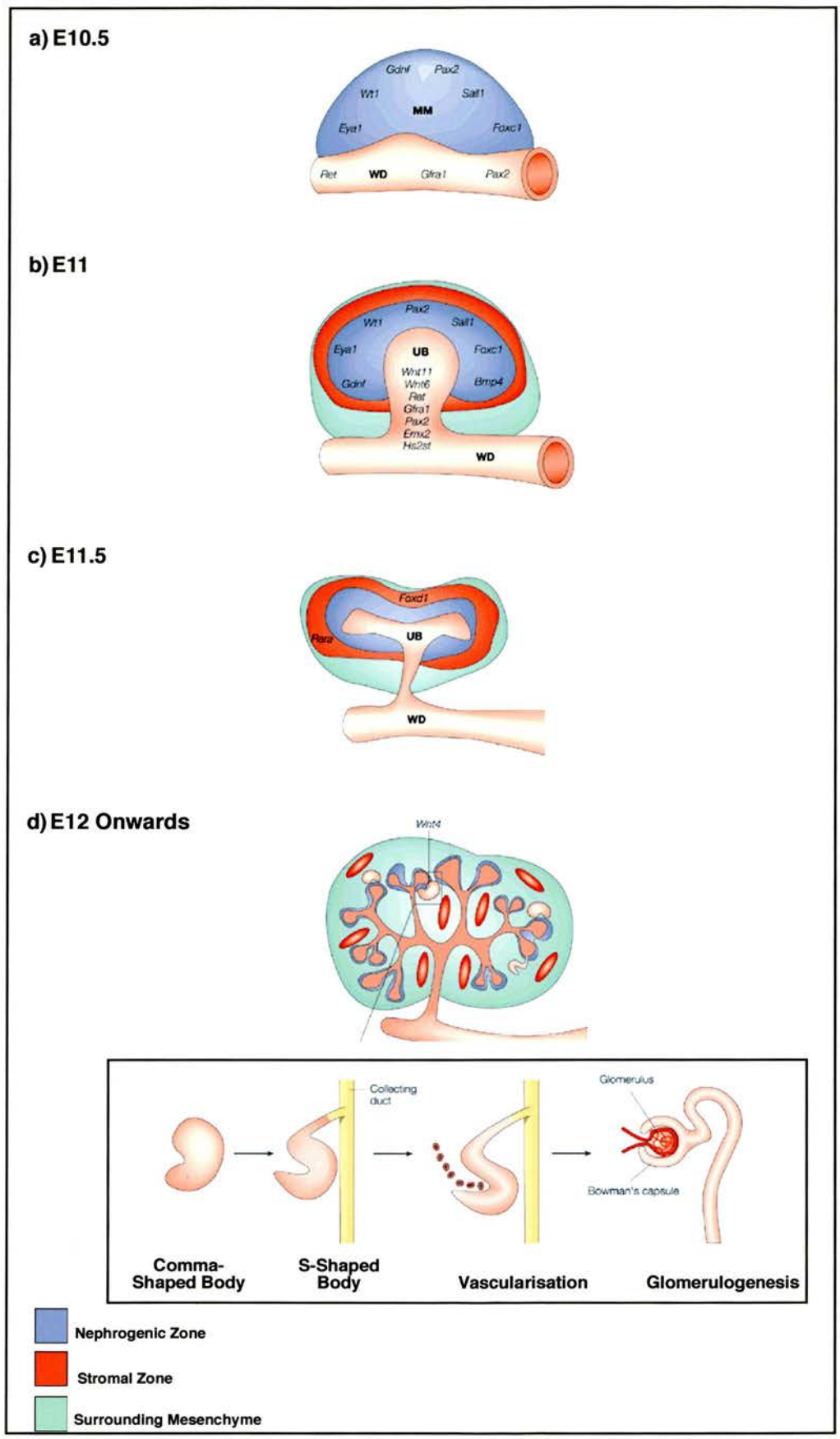


Figure 1.1 Kidney Development in the Mouse

a) The ureteric bud develops from the Wollfian duct at around E10.5 b) The ureteric bud grows into the metanephric mesenchyme. The mesenchyme surrounding the tips of the bud is induced to condense c) From E11.5 onwards, the condensed mesenchyme induces the ureteric bud to branch d) From E12.5 onwards, in association with ureteric branching morphogenesis, mesenchyme undergoes a mesenchymal-to-epithelial transformation to form the nephron. This occurs through the comma and s-shaped stages. The expression pattern of some of the genes involved in the process are shown (Figure taken from Vainio and Lin. 2002)

(Stewart *et al.*, 1992; Escary *et al.*, 1993). During nephrogenesis, the metanephric blastema undergoes an epithelial conversion via comma- and S-shaped structures to form a spherical cyst called the renal vesicle, which eventually matures to form the functional nephron (Figure 1.1 (d))

It is thought that Wilms' tumours arise from the pluripotent metanephric blastemal cells following the induction of differentiation as tumours often go on to develop tubular and glomerular-like structures (Hastie, 1994). A typical Wilms' tumour is classified as triphasic, consisting of undifferentiated blastema, epithelial structures, and stromatous components, all of which derive from the blastema during normal development of the kidney. Normally, in humans the metanephric mesenchyme has undergone conversion to epithelial structures by 36 weeks of gestation (Hastie, 1994). After this time, nephrogenesis ceases and any mesenchymal cells that have persisted and have failed to generate epithelium are thought to either give rise to stromal cells or undergo apoptosis. Any persistence of blastemal cells is abnormal and represents structures known as nephrogenic rests which are thought to represent precursor lesions to Wilms' tumour. Nephrogenic rests are found in 40% of unilateral and 100% of bilateral Wilms' tumour (Beckwith *et al.*, 1990). Therefore, it would appear that Wilms' tumour represents a malignancy in which its development is closely tied to the normal organogenesis of the kidney.

1.2 Wilms' Tumour Suppressor 1 (*WT1*) Gene

Considerable insights into the mechanisms underlying tumourgenesis have come from identifying tumour predisposition genes (Ponder, 2001). Clues to the chromosomal location of the first Wilms' tumour predisposition gene to be identified, *WT1*, came through the observation that Wilms' tumours often develop in children with a rare condition called aniridia, or lack of an iris. The close association between spontaneous aniridia and Wilms' tumour led to the isolation of a region on the short arm of chromosome 11. Patients presenting with a

chromosome 11p deletion syndrome, known as WAGR (Wilms' tumour, Aniridia, Genitourinary anomaly, mental Retardation) have constitutional hemizygous deletions of chromosomal region 11p13 (Francke *et al.*, 1979). High resolution molecular mapping on these constitutional deletions led to the identification and cloning of the Wilms' tumour predisposition gene, *WT1,* at the11p13 locus (Call *et al.*, 1990; Gessler *et al.*, 1990).

It was assumed that the gene would be a tumour suppressor gene, and as in the case of Rb1 in Retinoblastoma, inactivation of both copies of *WT1* would lead to tumourigenesis (Knudson, 1971). This was demonstrated to be the case when constitutional mutations within the *WT1* gene was detected in two individuals with Wilms' tumours and genital abnormalities (Pelletier *et al.*, 1991). Subsequently, it was also demonstrated that inactivation of *WT1* has been shown to occur in the nephrogenic rests, the precursors to Wilms' tumours (Park *et al.*, 1993). However *WT1* is deleted or mutated in only approximately 10% of sporadic Wilms' tumours, implicating a role for WT1 in the aetiology of the malignancy, but highlighting that the disease does not always conform to the simple two-hit model (Gessler *et al.*, 1994; Little and Wells, 1997). The complexity of Wilms' tumour could be explained by the presence of several other distinct Wilms' tumour suppressor genes. Additional genomic loci associated with sporadic Wilms' tumour have been mapped to chromosome 11p15, 16q, 3q, 4q, 9p and 20p (For a review see Menke *et al.*, 1998). However to date only the *WT1* gene at 11p13 has been cloned and shown to play a direct role in the aetiology of Wilms' tumour.

1.2.1 Structure of *WT1*

The *WT1* gene consists of 10 exons spanning 50 kb of genomic sequence and generates a transcript of around 3 kb (Call *et al.*, 1990; Gessler *et al.*, 1990; Haber *et al.*, 1991). Translation of the WT1 transcript reveals a protein that contains several motifs characteristic of a transcription factor. It encodes for a protein which

contains 4 zinc fingers of the Kruppel-type at the C-terminus, and at the N-terminus a proline/glutamine rich putative transactivation domain (Figure 1.2). These two motifs are features commonly found in *bona fide* transcription factors, such as EGR1 and SP1, and therefore suggested a transcriptional function for WT1. However, understanding the precise function of the *WT1* gene is complicated by the fact that it can potentially encode 24 protein isoforms

1.2.2 WT1 Protein Isoforms

The *WT1* gene encodes up to 24 different protein isoforms through a combination of alternative splicing, alternative translational initiation and RNA editing.

There are two alternatively spliced exons (Haber *et al.*, 1991). One alternative splicing event results in either inclusion or exclusion of exon 5 which encodes for 17 amino acids N-terminal of the four zinc fingers; the second event results from variation in the splice donor site used at the end of exon 9, resulting in the absence or presence of 3 amino acids (lysine-threonine-serine;KTS) between the third and fourth zinc finger.

Depending on the absence or presence of the two splice inserts, the WT1 proteins have a molecular mass of 52-54 kDa (Morris *et al.*, 1991). Added to the alternative splice forms, there is also an alternative non-ATG translational initiation site (CTG) which is located 204 bp upstream of the major ATG start site. This produces WT1 isoforms that have an additional 68 amino acid N-terminal extension and a molecular mass of 60-62 kDa (Bruening *et al.*, 1996).

The WT1 mRNA is also edited. In adult rat kidneys and testis, as well as in adult human testis, a thymidine residue in exon 6 of WT1 (position 839 in rat and position 1222 in human) is changed to a cytosine residue (Sharma *et al.*, 1992; Sharma *et al.*, 1994). Within the WT1 protein this leads to an isoleucine to proline amino acid alteration. This modification appears to affect the transcriptional

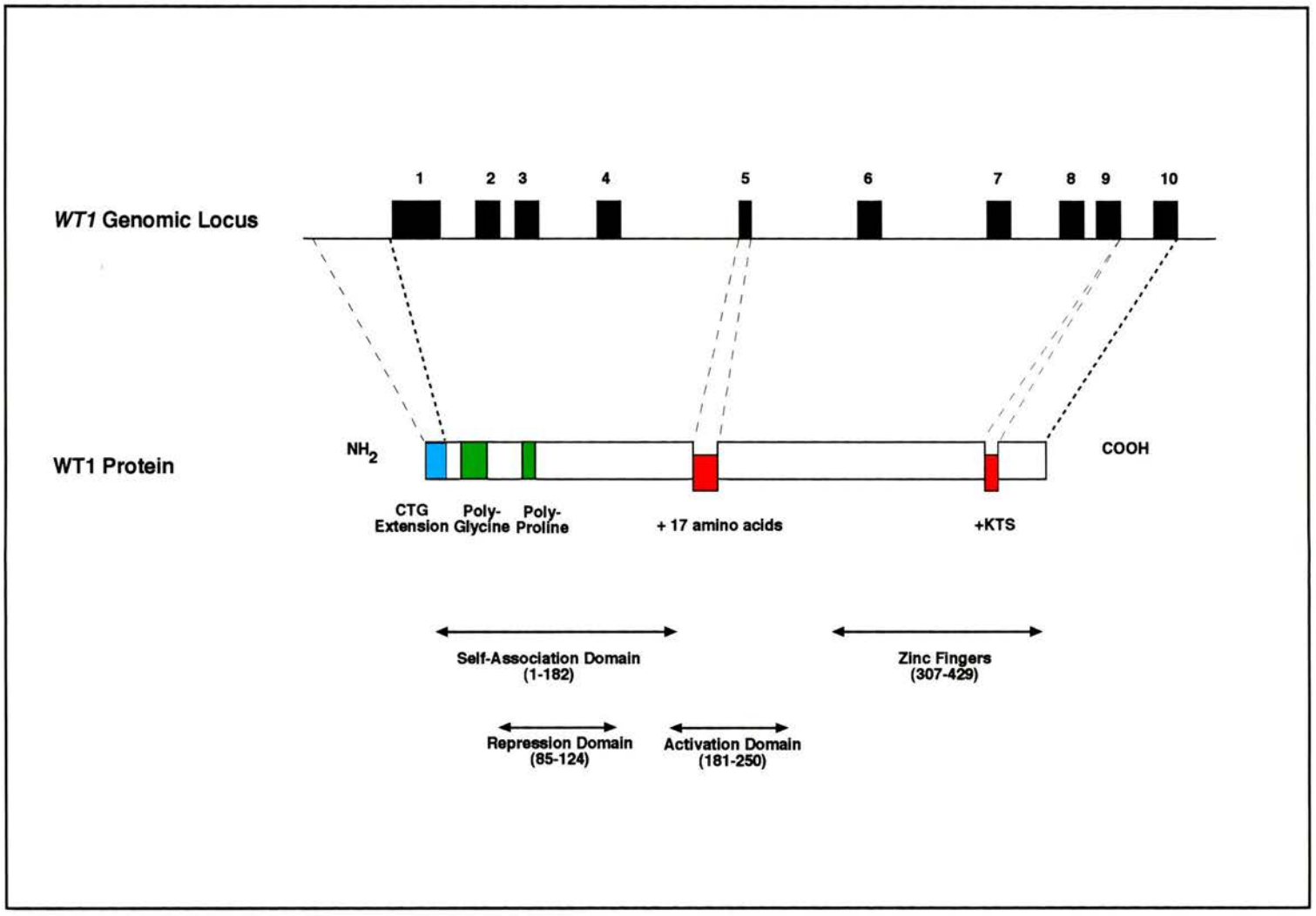


Figure 1.2 Schematic Representation of *WT1* Gene and WT1 Protein Structure

activity of the isoform. The edited isoform represses transcription from the EGR1 promoter 25-30% less efficiently that the unedited version (Sharma *et al.*, 1994). With regards to its biological significance *in vivo*, it appears that RNA editing is not a mechanism by which WT1 is altered during tumourgenesis, as the analysis of primary Wilms' tumours did not detect RNA editing of WT1 in the specimens (Gunning *et al.*, 1996). Therefore, the biological effects of RNA editing in the WT1 mRNA remains unclear.

1.2.3 Evolutionary Conservation of Protein Isoforms

So are all of these WT1 isoforms important? WT1 has been cloned from a variety of different organisms. Comparison of *WT1* sequence from mouse, rat, chick, alligator, xenopus, zebrafish and fugu reveals extensive conservation across species, suggesting an important role for the gene throughout vertebrate evolution (Sharma *et al.*, 1992; Kent *et al.*, 1995; Miles *et al.*, 1998). With regards to the particular motifs of WT1, across the zinc fingers, between human, mouse and rat, there is 100% amino acid sequence identity. In all the isolated *WT1* sequences from vertebrates, there is conservation of the +KTS alternative splice form (Kent *et al.*, 1995). In fugu, there is an alteration in the KTS amino acid sequence, with a proline replacing the threonine to produce KPS instead of the KTS. The biological significance of this amino acid change from KTS to KPS is unknown, however the KPS is still alternatively spliced (Miles *et al.*, 1998). Overall, the conservation of the KTS alternative splice variant emphasises the importance of this splice form in vertebrate development. The conservation of these two isoforms over 450 million years of evolution suggests that they serve distinct functions in development. Recently, the development of mouse models that produce only KTS or –KTS insert confirmed that the KTS isoforms are crucial during urogenital development (Hammes *et al.*, 2001) (See section 1.7.4).

In contrast, the 17 amino acid alternative splice site, the proline/glutamine rich domain, the alternative CTG start site and the occurrence of RNA editing are not conserved between all vertebrates, being present only in mammals (Sharma *et al.*, 1992; Kent *et al.*, 1995; Miles *et al.*, 1998). Therefore, does the increased complexity of WT1 in mammals indicate a role for WT1 in more mammalian-specific developmental processes? The recent mouse model knockouts of the mammalian-specific isoforms would indicate otherwise (See section 1.7.5)

1.3 Functions of WT1

1.3.1 WT1 as a Transcription Factor

WT1 has many structural properties that are typical of a transcription factor, including a glutamine/proline rich N-terminus, nuclear localisation signal and four zinc fingers with a Kruppel-type motif. The zinc fingers 2, 3 and 4 of WT1 share close structural homology with the three zinc fingers of EGR1. The WT1 zinc finger domain has been shown to bind to the characteristic CG-rich EGR1 DNA-binding element, although with a lower affinity than EGR1 (Rauscher *et al.*, 1990). A second potential WT1 DNA-binding site motif has been identified as containing TCC repeats (Wang *et al.*, 1993 (a)). This motif has been mapped to the WT1-responsive promoters of PDGF-A and also EGFR. A more refined and specific WT1 binding site, termed WTE, has been identified by PCR selection of genomic DNA sequences with high affinity for WT1, coupled with extensive mutational studies (Nakagama *et al.*, 1995). WT1 demonstrates a 20-30 fold higher affinity for the WTE binding site than EGR1 and has been identified in the promoters of amphiregulin and SF-1, which appear to be physiologically authentic WT1 target genes (Lee *et al.*, 1999; Wilhelm *et al.*, 2002).

Although the zinc fingers of WT1 confer sequence-specific DNA-binding activity, the presence of the KTS insert between zinc fingers 3 and 4 greatly effects the

binding affinity of WT1 for DNA (Rauscher *et al.*, 1990). Initially, it was thought that only the -KTS isoforms could bind to the EGR1 consensus DNA-binding site. Subsequently further studies identified DNA sequences that both -KTS and +KTS could bind to, although with different affinities (Bickmore *et al.*, 1992). WT1 -KTS and +KTS bound to the same overlapping DNA sequence of the IGFII, PDGF-A and Pax2 genes. Recently, in a study of IGFII gene regulation by WT1, the isoforms were found to have opposite effects, with the –KTS activating the IGFII P3 promoter, whilst the +KTS represses its activity (Caricasole *et al.*, 1996). Therefore, the KTS splice variants appear to exhibit differential transcriptional regulation of the same target genes.

Studies using nuclear magnetic resonance (NMR) techniques have investigated the structural consequences of insertion of the KTS sequence between zinc fingers 3 and 4, both in free solution and complexed with DNA (Laity *et al.*, 2000). The NMR spectra indicated that the two isoforms are nearly identical in structure in the absence of DNA. However, upon binding to DNA it was shown that the KTS insertion disrupts important interactions of the linker region and other zinc fingers, lowering the stability of the zinc finger/DNA complex. This provides structural information relating to the observed alternative affinity of the -/+ KTS isoforms for DNA.

1.3.2 Transcriptional Targets of WT1

What are the target genes that WT1 transcriptionally regulates? As described above the identification of potential WT1-specific DNA-binding motifs has led to the identification of numerous putative WT1 target genes which contain WT1 binding sites within their promoters. Extension of these findings has shown that the promoters of these potential WT1 target genes can be modulated by WT1 in transfections assays (Table 1.1) (For a review see Reddy and Licht, 1996; Lee and Haber, 2001). It is evident from these *in vitro* reporter assays that WT1 can mediate

Growth Factors

IGF-II
PDGF-A
CSF-1
TGF-β
Amphiregulin
Inhibin-α
Midkine
MIS
CTGF

Growth Factor Receptors

Insulin Receptor
IGF-I Receptor
EGFR
RAR-α

Transcription Factors

FREAC-4 (FoxD1/BF-2)
Egr-1
WT1
C-Myb
C-Myc
N-Myc
Pax2
Sry
SF-1

Others

Syndecan-1
E-Cadherin
IGFBP-4
NovH
ODC
Hsp70
p21
Bcl-2
HTERT

Table 1.1 Published WT1 Target Genes

(Compiled from the reviews by Reddy and Licht (1996) and Lee and Haber (2001))

both transcriptional repression and activation. The list of potential target genes described in the literature as being either transcriptionally activated or repressed by WT1 has been established mainly through two *in vitro* approaches.

The first approach has employed the use of WT1 promoter-reporter constructs and electrophoretic mobility shift assays (EMSA). This approach follows a strategy in which the promoter region of a potential WT1-regulated gene is co-transfected into heterologous cell lines along with a WT1 expressing plasmid. The transcriptional repression or activation is assayed to ascertain whether WT1 has an effect upon the regulation of this gene. EMSA is then used to identify potential WT1 consensus sequences within that promoter. However the interpretation of the data from these assays is difficult. Various factors, including the number of WT1 binding sites within the promoter, the cell type used in the experiment, the choice of expression vector and also the particular isoform of WT1 employed in the assay, have all been shown to affect the outcome of this assay. For example, the promoter of the EGR-1 gene is activated by WT1 in Saos-2 and U20S, human tumour cell lines, whilst WT1 represses the same promoter in mouse NIH3T3 cells (Maheswaran *et al.*, 1993; Englert *et al.*, 1995).

The second approach has been to establish cell lines overexpressing single WT1 isoforms and then screen for changes in gene expression profiles between expressing and non-expressing cell lines. Following this methodology, U2OS osteosarcoma cell lines with tetracycline-inducible expression of –KTS and +KTS WT1 isoforms were screened for altered gene expression profiles using oligonucleotide microarrays containing 6800 human transcripts (Lee *et al.*, 1999). From this analysis, amphiregulin (a member of the epidermal growth factor family) was shown to be up-regulated following expression of the –KTS isoform (Lee *et al.*, 1999). *In vitro*, the WT1 -KTS isoform was shown to bind directly to the amphiregulin promoter through the presence of a WTE binding element, resulting in transcriptional activation of amphiregulin. Immunohistochemistry and RNA *in*

situ hybridisation studies reveal that WT1 and amphiregulin, *in vivo*, share the same temporal and cell type-specific expression pattern in the developing kidney. It was also demonstrated that in embryonic kidney rudiments, recombinant amphiregulin stimulates epithelial differentiation *in vitro*. Therefore these observations suggest that amphiregulin is a physiologically relevant target for transcriptional control by WT1. However, apart from amphiregulin, no other previously reported WT1 target genes were altered in this *in vitro* system.

Utilising the same tetracycline inducible system described above, a rat embryonic kidney cell line, derived from the undifferentiated renal mesenchyme using SV40 large T antigen, was used to identify potential WT1 targets (Palmer *et al.*, 2001). Cells derived from the embryonic kidney were chosen to enable the identification of more physiological relevant targets for WT1 regulation. From this study, the authors identified podocalyxin, a major structural membrane protein component of the podocytes as being regulated by WT1 (Palmer *et al.*, 2001). Characterisation of the promoter demonstrated the presence of WTE elements. Expression analysis of podocalyxin in the developing kidney was shown to mirror that of WT1 (Palmer *et al.*, 2001). This system confirmed transcriptional regulation of amphiregulin by the –KTS isoform, however it was unable to detect any changes in the expression of other reported candidate genes (EGR1, IGF-II, PDGF-A, Bcl-2, IGFR, C-Myc and EGFR).

Overall, the approach of using cell lines expressing single isoforms has its limitations. Originally, the WT1 over-expression studies usually employed cell lines that were not physiologically relevant to the *in vivo* expression pattern of WT1 i.e. these cell types do not normally express WT1 (Englert *et al.*, 1997; Hosono *et al.*, 1999; Lee *et al.*, 1999). Although successful at identifying amphiregulin, other more genuine WT1 target genes may have been missed. It is also unclear whether the alteration of gene expression following over-expression of single WT1 isoforms reflects the situation *in vivo*, where normal physiological concentrations and ratios

of splice forms of WT1 are present. The correct ratio of different WT1 isoforms has been shown to be crucial in development and disease. This is most evident in the expression of the KTS isoforms, as demonstrated by Frasier syndrome patients and shown in the KTS mouse models (Hammes *et al.*, 2000). Therefore, although providing interesting and potential WT1-regulated targets, a more physiologically relevant system is required for the study of WT1 in the transcriptional control of gene expression.

In an attempt to tackle these issues, there has been a move towards the use of more developmentally-appropriate cell lines which promote the identification of legitimate downstream gene targets. These cell lines will possess the correct cofactor combination required for the proper interaction of WT1 to target genes. Added to this, assays have also been developed which allow the identification WT1 transcriptional targets by "knocking-down" WT1 in cells lines that endogenously expresses this gene. In this approach, a dominant-negative isoform of WT1 is used to inhibit the function of the endogenous wild-type protein. Dominant-negative WT1 mutations are found in patients with Denys-Drash syndrome (Pelletier *et al.*, 1991). The DDS mutations all lie within the zinc fingers of WT1, and ablate the ability of WT1 to bind to DNA and function as a transcriptional regulator (Pelletier *et al.*, 1991; Englert *et al.*, 1995). Furthermore, since WT1 self-associates, it is assumed that the mutant protein is capable of blocking the activity of the wild-type protein, thereby acting in a dominant negative fashion (Holmes *et al.*, 1997).

Employing this approach, a polyoma-transformed mouse mesonephric cell line, M15, expressing a dominant-negative DDS isoform of WT1 (M15 DDS), was screened for altered gene expression in comparison to wild-type M15 cells lines using nylon based cDNA macroarrays (Sim *et al.*, 2002). Comparison of the expression profiles of M15 and M15 DDS clones identified Wnt-4 as being down-regulated in the M15 DDS cells. While several potential WT1 binding sites were detected in the Wnt-4 promoter, no evidence could be gathered to establish whether

the regulation of Wnt-4 by WT1 was direct (Sim *et al.*, 2002). The significance of an interaction with Wnt-4 is discussed below. Again, further analysis of the expression of some of the reported WT1 candidate genes failed to detect any change in their expression (Bcl-2, C-Myb, C-Myc, CSF-1, EGR1, IGF2, IGFR1, PDGF-A, RARα, and TGFβ1).

Using the same approach of stably expressing a WT1 DDS isoform, but this time in a Wilms' tumour cell line, connective tissue growth factor (CTGF) was identified as being a potential WT1-regulated transcriptional target (Stanhope-Baker and Williams, 2000). The authors assessed the validity of CTGF as a WT1 target by promoter-reporter assays. In these assays, they showed that the CTGF promoter is repressed by WT1. The physiological significance of CTGF regulation by WT1 is enhanced by the fact that CTGF shares homology to NovH, a gene that has been postulated to be potential WT1 target (Martinerie *et al.*, 1996). CTGF and NovH contain a domain that is commonly present in the family of proteins called the Insulin-Like Growth Factor Binding Proteins (IGFBPs). It is interesting to note that a recent study has suggested that WT1 may regulate two members of the IGFBP family, IGFBP-3 and IGFBP-4 (Wagner *et al.*, 2001) (Discussed in Chapter 5). These proteins modulate the biological activity of the insulin like growth factors, IGF-I and IGF-II. Since IGF-II is over-expressed in a subset of Wilms' tumours, this proposed regulation of CTGF by WT1 may play a role in the tumourgenesis and disease progression in patients with WT1 mutations (Cui *et al.*, 2001).

In summary, whilst many potential transcriptional target genes have been identified for WT1, there are few for which there is compelling evidence to indicate that they are genuine physiological targets *in vivo*. In only one case, that of SF-1, has genetic and transgenic studies been carried out *in vivo* to support the idea that it is a direct target gene for WT1 (Wilhelm *et al.*, 2002).

1.3.3 WT1 in RNA Metabolism

Although compelling evidence exists for WT1 acting as a transcription factor, recent data also supports a role for WT1 in post-transcriptional processes. The initial evidence that WT1 may act as a post-transcriptional regulator came from the study of the subnuclear localisation of the WT1 in cell lines and tissues which endogenously express WT1 (Larsson *et al.*, 1995). In this study, a proportion of WT1 was concentrated in nuclear 'speckles' that correlated with the expression pattern of proteins involved in RNA splicing. The authors demonstrated that WT1 co-immunoprecipitated with known splicing factors including U1-70K, U2-B" and P80 coilin. When individual isoforms of WT1 were over-expressed in a cell line negative for endogenous WT1, the +KTS isoforms preferentially associated with nuclear speckles, whilst the –KTS isoforms were spread diffusely throughout the nucleus and in areas where the classical transcription factors, TFIIB and SP1, are abundant. These results were reproduced and extended to show that the subnuclear localisation of WT1 +KTS was RNase, but not DNase, sensitive (Englert *et al.*, 1995, Caricasole *et al.*, 1996). Using a variety of biochemical techniques, it was demonstrated that WT1 was enriched by oligo(dT) chromatography, along with the splicing proteins U2AF65 and hnRNP A1, and the U5 small nuclear RNP-associated protein, p116 (Ladmomery *et al.*, 1999). Gel filtration and sedimentation profiles suggested that WT1 was present in the RNase-sensitive fractions. This suggested that WT1 protein is commonly found to be associated with nuclear poly (A)+ RNP.

Further work focusing upon the potential role of WT1 in post-transcriptional processes demonstrated that WT1 interacts with the ubiquitous splicing factor, U2AF65 (Davies *et al.*, 1998). This interaction, characterised by using a two-yeast hybrid approach coupled with *in vitro* binding assays and co-immunoprecipitation, demonstrated that the +KTS isoforms preferentially associated with U2AF65. WT1 has also been shown to interact with a novel protein, WTAP (WT1-associated

protein). WTAP is the mammalian orthologue of the *Drosophila* gene, *fl(2)d*, which is required for female specific splicing of *Sxl* and *Tra* pre-mRNAs in the *Drosophila* sex determination pathway (Little *et al.*, 2000; Penalva *et al.*, 2000). Both of these proteins are components of the splicing machinery, and the association of WT1 with these proteins links WT1 directly to a role in RNA splicing.

Following the observed association of WT1 +KTS isoforms with the splicing machinery, computer modelling was used to postulate the existence of an RNA recognition motif (RRM) in the N-terminal domain of WT1. This hypothetical model suggested that this domain shared structural homology to the RNA recognition motif of the splicing factor U1A (Kennedy *et al.*, 1996). Therefore, mounting data suggests that WT1, apart from its role as a transcription factor, is also is involved in post-transcriptional functions and specifically may play a role in RNA splicing.

If WT1 does actually bind RNA *in vivo*, what are the specific RNA target transcripts? The only RNA sequence identified as a target for WT1 is located in exon 2 of the IGFII mRNA, and is bound by both –KTS and +KTS isoforms, with the +KTS isoforms binding with a higher affinity (Caricasole *et al.*, 1996). More recently, Bardeesey and Pelletier identified three groups of RNA ligands that bound to WT1 from a random ligand pool (Bardeesy and Pelletier, 1998). Using EMSAs, they found that zinc fingers 2-4 were responsible for recognition of the RNA targets by WT1, and that the presence of the KTS insert dramatically decreased the affinity of WT1 for the RNA ligands. Whilst this method identified several sequences, the significance of these sequences remains to be established.

In summary, WT1 appears to have two distinct regulatory functions, with -KTS isoforms involved in transcriptional control, whilst +KTS isoforms influence post-transcriptional control. Taken together, the distinct nuclear localisation of the WT1

+KTS isoforms, the coelution of WT1 with poly(A)+ cellular fractions, and the association of WT1 with splicing proteins U2AF65 and WTAP, have raised the possibility that this isoform may mediate some aspect of mRNA processing. However further studies are required to demonstrate a clear role for WT1 in post-transcriptional RNA processing also to identify physiologically relevant mRNA targets.

1.4 Cellular Functions of WT1

What does WT1 do at the cellular level? Studies have indicated that WT1 has a role in growth suppression and apoptosis.

Observations from gain-of-function experiments point to a role for WT1 in growth suppression. The importance of WT1 in the control of cell growth was demonstrated when isoforms of WT1 were introduced into a Wilms' tumour cell line expressing an aberrantly spliced WT1 transcript. This resulted in the suppression of proliferation (Haber *et al.*, 1993). It was also demonstrated that microinjection of WT1 cDNA into NIH3T3 cells synchronised in early G1 stage, blocked cell cycle progression into S phase (Kudoh *et al.*, 1995). This phenomenon was also observed using CV-1, Cos-7, F9 or P19 cell lines. Another study found that over-expression of the WT1 (-KTS) isoform in Saos-2 cells and U2O2 cells resulted in a 25% increase in cells arrested in the G1 cell cycle phase arrest (Englert *et al.*, 1995). This cell cycle effect was shown to correlate with transcriptional activation of p21.

WT1 loss-of-function experiments using WT1 antisense oligos have demonstrated that suppression of endogenous WT1 expression in K562 cells induces G2/M arrest (Algar *et al.*, 1996; Yamagami *et al.*, 1998). Therefore, WT1 may therefore play a role in the control of two cell cycle checkpoints: WT1 over-expression induces G1/S arrest whilst WT1 knockdown induces G2/M arrest.

In WT1 null mice, the mesenchymal cells of the kidney blastema fail to undergo differentiation and degenerate via apoptosis indicating a crucial requirement for WT1 for their survival (Kreidberg *et al.*, 1993). WT1 has also been shown to suppress P53-induced apoptosis (Maheswaran *et al.*, 1995) suggesting a role for WT1 in promoting cell survival. In contrast, ectopic expression of WT1 can induce apoptosis in a number of cells lines (Englert *et al.*, 1995; Menke *et al.*,1997; Murata *et al.*, 1997; Smith *et al.*, 1998).

The work in various cell systems has indicated a role for WT1 in growth suppression and regulation of apoptosis (For review see Little *et al.*, 1999; Lee and Haber, 2001). Although the results do provide some clues to the function of WT1 at the cellular level, variability between cell lines and the variation of effects when over-expressing single isoforms of WT1 indicates that a more physiologically relevant system is required. Also conclusions based upon cancer-derived cell lines is limited, as these cells lines are often restricted in their ability to respond to potential cellular differentiation signals. Therefore recently several groups have used pluripotent cell lines to study WT1. One of the most interesting is the differentiation of embryonal carcinoma and embryonic stem cells with retinoic acid (Scharnhorst *et al.*, 1997). The treatment of F9 and P19 embryonal carcinoma cells with retinoic acid triggers differentiation into endodermal (F9) or endodermal and ectodermal (P19) and is accompanied by induction of endogenous WT1 proteins (Kudoh *et al.*, 1996; Scharnhorst *et al.*, 1997). Therefore this system could be used to ascertain what happens in cells that endogenously express all WT1 isoforms in their strictly defined physiological ratio and what effect loss-of-function for WT1 would have on this system.

1.5 Expression of WT1 during Development

The expression pattern of WT1 has been analysed in detail by a number of authors during mouse and human embryonic development in an attempt to gain further

insights into its role in development (Pritchard Jones *et al.*, 1990; Armstrong *et al.*, 1993; Rackley *et al.*, 1993; Moore *et al.*, 1998).

1.5.1 WT1 Expression during Kidney Development

WT1 expression is detected throughout the three stages of kidney development. WT1 is first detected in the nephrogenic mesenchyme of the intermediate mesoderm prior to the formation of the pronephros at E8.5 in mouse (Pritchard Jones *et al.*, 1990; Armstrong *et al.*, 1993). With the formation of the mesonephros, the cellular caps of the mesonephric ducts express WT1. As the ureteric bud develops from the mesonephric duct, the metanephric mesenchyme expresses low levels of WT1. However as the metanephric blastema condenses around the ureteric bud, an up-regulation of WT1 expression occurs in the blastema. During the mesenchymal-to-epithelial conversion to form the renal vesicle, WT1 expression increases and continues in the renal vesicle. The renal vesicles then undergo a series of morphological changes as they differentiate to form epithelial structures known as comma-shaped and S-shaped bodies. During this process, WT1 expression becomes localised to the cells at the proximal part of the S-shaped body (Pritchard-Jones *et al.*, 1990). These cells flatten to form the podocytes of the presumptive glomerulus. WT1 expression within the metanephric kidney becomes restricted to the podocytes of the glomeruli, where it remains expressed throughout postnatal development and continues in adulthood. The remaining structures of the nephron, including the proximal and distal convoluted tubules and the loop of Henle, do not express WT1

After the induction of the metanephric mesenchyme by the ureteric bud, at least two separate cell populations are found in the mesenchyme. In close proximity to the ureteric bud are a tubular epithelial population of cells within the nephrogenic zone. More peripheral to the ureteric bud is the stromal cell population. Within this context, cells of the metanephric mesenchyme that are not induced, or do not

undergo nephrogenesis, form either the supporting stroma of the kidney or undergo apoptosis. As the blastemal cells of the metanephric mesechyme undergo transition to form the stromal component of the kidney, WT1 expression is lost (Prichard-Jones *et al.*, 1990; Armstrong *et al.*, 1993)

1.5.2 WT1-Regulated Genes involved in Kidney Development

The involvement of several developmentally-regulated proteins in nephrogenesis and their relation to WT1 has been established. Work from mouse models has demonstrated that WT1 is required at multiple stages throughout kidney development, from the induction of the ureteric bud to the formation of the nephron (see section 1.7). The fundamental role of WT1 in kidney development has been highlighted by the failure of WT1 null mice to develop metanephric kidneys (Kreidberg *et al.*, 1993). There is no induction of the ureteric bud to branch from the mesonephric duct and this leads to the cells of the metanephric mesenchyme to undergo apoptosis. The apoptosis of the blastemal cells in the WT1 null mice may result either from a direct survival signal effect of WT1 itself or, indirectly, as a consequence of disrupted survival signals from the ureteric bud.

The absence of WT1 may result in either the up- regulation of apoptosis-inducing factors or the down-regulation of survival factors. With this in mind, WT1 has been shown to transcriptionally activate the expression of Bcl-2, which encodes an anti-apoptotic protein (Mayo *et al.*, 1999). Bcl-2 is expressed in the normal undifferentiated metanephric mesenchyme, and therefore the absence of WT1 in the blastemal cells may lead to possible down-regulation of Bcl-2, and hence lead to the observed apoptosis of these cells. Support for Bcl-2 being a potential target gene of WT1 during early nephrogenesis is the observation that Bcl-2 knockout mice have an aberrant kidney phenotype. Bcl-2 knockout mice have smaller kidneys, which contain fewer nephrons, and the metanephtic blastema displays a greater susceptibility to apoptosis (Novack and Korsmeyer, 1994).

In the condensing mesenchyme, WT1 may promote kidney development by transcriptionally inducing amphiregulin (Lee *et al.*, 1999). Due to the overlapping expression pattern of amphiregulin and WT1 during nephrogenesis, it is possible that activation of amphiregulin by WT1 facilitates ureteric bud branching at the earliest stages of nephrogenesis (Lee *et al.*, 1999). However amphiregulin is not the sole factor involved in branching of the ureteric bud as the mouse knockout of amphiregulin does not exhibit aberrant kidney development, indicating that other factors are also involved in this process (Luetteke *et al.*, 1999).

Recently Wnt-4 has been identified as a potential WT1-regulated gene (Sim *et al.*, 2002). During mouse metanephric kidney development, Wnt-4 expression is first detected in the metanephric mesenchyme that condenses around the tips of the invading ureteric bud. Wnt-4 expression continues during the formation of the comma and S-shaped bodies but is lost when fusion of the epithelial tubules to collecting ducts occurs (Stark *et al.*, 1994). Mice homozygous null for Wnt-4 exhibit small agenic kidneys, consisting of undifferentiated mesenchyme interspersed with branches of collecting duct epithelium. This indicates a crucial role for Wnt-4 in the transition of the mesenchyme to epithelium that occurs during the development of the nephron. Therefore, this links WT1, via regulation of Wnt-4, to the mesenchymal-epithelial transition during the early developmental stages of metanephric kidney formation.

1.5.3 WT1 Expression in Gonadal Development

In mammals, the gonads and kidney are derived from the intermediate mesoderm and a common precursor, the urogenital ridge. Gonadal development in the mouse begins as a thickening of the coelomic epithelium of the urogenital ridge, known as the primary sex cords at E11. This thickening of the epithelium marks the first stage of gonadal development, which is followed by development of a prominent gonadal ridge at around E12. This first step of gonadal development establishes the

bipotential, or indifferent, gonad. This indifferent gonad has the capacity to differentiate into completely separate sexual structures, the ovary or the testis.

WT1 is expressed in the proliferating cells of coelomic epithelium of the urogenital ridge and is also present in the underlying mesenchyme of the gonadal ridge. At around E13 in mouse, sexual differentiation of male and female gonadal development begins. In the ensuing development of the gonad, WT1 expression becomes restricted to the Sertoli cells of the testis, and the granulosa cells and surface epithelium of the ovaries (Armstrong *et al.*, 1993). Expression in the Sertoli and granulosa cells continues into adult life, with expression of the gene within these cells related to the state of maturation of their associated germ cells (Armstrong *et al.*, 1993; Rackley *et al.*, 1993; Moore *et al.*, 1998).

1.5.4 Other areas of WT1 expression

In addition to the expression in the developing urogenital system, WT1 is also expressed in the mesothelium lining of organs within the thoracic and abdominal cavities, including the surface of the diaphragm, gut mesenteries and the inner surface of the coelomic cavity (Armstrong *et al.*, 1993; Rackely *et al.*, 1993; Moore *et al.*, 1998). In the wall of the thoracic cavity, WT1 is expressed in the cells adjacent to differentiating muscle in the intercostal region, and within the developing musculature of the body wall (Moore *et al.*, 1998). Further expression was also detected in the early pro-epicardium, the epicardium and the sub-epicardial mesenchymal cells of the heart (Moore *et al.*, 1998). WT1 is also expressed at high levels in the spleen.

Other sites of expression include discrete pockets of staining in the nervous system. WT1 expression has been detected in the spinal cord and also in the ventricles of the brain (Armstrong *et al.*, 1993; Rackely *et al.*, 1993; Moore *et al.*, 1998). WT1 expression has also been detected in the haematopoietic system, with expression

detected in early haematopoietic precursors in the bone marrow (King-Underwood *et al.*, 1996; Maurer *et al.*, 1997; Baird and Simmons, 1997).

1.6 The Involvement of WT1 in Disease

The identification of WT1 mutations in human patients has provided important insights into the functions of WT1. Developmental defects present in patients suffering from WAGR, Denys-Drash and Frasier syndrome has demonstrated a fundamental role for WT1 in the development of the urogenital system. Denys-Drash syndrome (DDS) and Frasier syndrome (FS) are two related conditions caused by mutations of the WT1. Both syndromes are characterized by male pseudohermaphroditism, a progressive glomerulopathy, and the development of genitourinary tumours. DDS and FS have previously been distinguished by differences in nephropathy, with DDS patients demonstrating diffuse mesangial sclerosis (DMS) in contrast to focal and segmental glomerulosclerosis (FSGS) in FS patients.

Denys-Drash syndrome (DDS) is a rare congenital childhood syndrome that includes symptoms of diffuse mesangial sclerosis, partial gonadal dysgenesis and a predisposition to Wilms' tumour. Patients suffering from DDS have been shown to be constitutionally heterozygous for WT1 point mutations (For review see Little and Wells, 1997). Analysis of the point mutations show that 92% of the WT1 mutations in DDS are present in the zinc finger regions. Most of the mutations are missense mutations within exon 8 and 9, which code for zinc finger domains 2 and 3. All mutations alter the structure of the DNA binding domain by disrupting the conformation of the zinc finger regions of WT1.

Frasier syndrome (FS) is caused by mutations in the splice donor site in intron 9 of one WT1 allele, resulting in the loss of expression of the WT1 +KTS isoforms (Barbaux *et al.*, 1997; Klamt *et al.*, 1998). This effects the balance in ratios of the

KTS isoforms, leading to the severe developmental defects. Although FS patients generally only develop glomerulopathy of the kidney and not Wilms' tumour, there has been one reported case of Wilms' tumour in a patient with FS (Barbosa *et al.*, 1999).

WAGR patients have a constitutional heterozygous deletion of chromosome 11p13 (Francke *et al.*, 1979). Children inheriting these deletions suffer from Wilms' tumour, aniridia, mental retardation, and boys in particular, suffer from mild pseudohermaphroditism; hence the full syndrome is known as WAGR (Wilms' tumour, Aniridia, Genitourinary abnormalities, Mental retardation).

Given the expression pattern of WT1 in a range of different tissues, it seems likely that the gene may also be involved in the development of a range of tumour types. WT1 expression has been detected in haematopoetic progenitors, suggesting a role for WT1 in early haematopoetic development. In WAGR patients, there is an increased rate of leukaemia as a secondary malignancy, and it has been shown that a WT1 mutation was found in the remaining allele of one WAGR patient with leukaemia (Pritchard-Jones *et al.*, 1994; Hartley *et al.*, 1994). WT1 mutations have been shown to be involved in the development of some types of leukemias. It has been shown that heterozygous WT1 mutations are present in approximately 15% of acute myloid leukaemias (King-Underwood and Pritchard-Jones, 1998).

The mesothelium is another tissue in which WT1 is expressed (Armstrong *et al.*, 1993). However examination of 32 cases of malignant mesotheliomas identified only one sample with a WT1 mutation present (Park *et al.*, 1993 (b)). Recent evidence points to a possible role for WT1 in the etiology of breast cancer. Using immunohistochemistry it has been demonstrated that WT1 expression is present in normal breast tissue, localised to the myoepithelial cells that are present in the ducts of the breasts (Silberstein *et al.*, 1997). Analysis of breast tumours by the same authors observed a reduction or absence of WT1 in 60% of breast tumours. Using

RT-PCR they also reported that WT1 isoforms lacking exon 5 and KTS were specifically expressed in tumours. The conclusion of this study was that the loss of WT1 correlated with tumourigenesis (Silberstein *et al.*, 1997). Contrary to these findings, another group has demonstrated that WT1 is not expressed in normal breast epithelium, but that it is highly expressed in the majority of primary breast tumours analysed (27 of 31 tumours) (Loeb *et al.*, 2001).

1.7 Mouse Models of WT1

1.7.1 WT1 Knockout Mice

To determine the role of WT1 *in vivo*, Kreidberg *et al* generated a mouse which lacked the first exon and 0.5kb of upstream sequence of WT1, removing the translational start site and producing a null mutation (Kreidberg *et al.*, 1993). On a 129/C57BL/6 background, adult heterozygous mice appeared phenotypically normal after 10 months of age. The homozygous WT1 null mice however exhibited severe abnormalities in the development of the kidney and gonads (Kreidberg *et al.*, 1993).

In WT1 knockout mice there is an arrest in the development of metanephric kidney. Specifically, at E11 the ureteric bud, which induces the metanephric mesenchyme, does not branch from the mesonephric duct. Furthermore whilst the metanephric mesenchyme forms, it undergoes apoptosis (Kreidberg *et al.*, 1993). WT1 null metanephric mesenchyme was assayed *in vitro* to see whether spinal cord explants, strong inducers of mesenchyme induction, could rescue the WT1 phenotype and induce the metanephric mesenchyme. Mesenchyme from the WT1 null mice failed to undergo induction, whilst wild-type mesenchyme did undergo induction. This demonstrates that WT1 is crucial for the cell autonomous differentiation of the metanephric blastema.

Therefore, the analysis of WT1 null mice indicates that the presence of WT1 is required for two steps during the first stages of kidney development. Firstly, its expression in the undifferentiated mesenchymal cells is required to initiate signals from the metanephric mesenchyme that promotes ureteric bud invasion. Secondly its expression the metanephric mesenchyme is required for either the survival of, or the reception of a survival signal, by the metanephric mesenchyme from the ureteric bud.

Alongside the failure of kidney development, WT1 null mice fail to develop gonads. Analysis of gonadal development in the WT1 null mice indicates that the thickening of the epithelium of the urogenital ridge is markedly reduced and the gonadal ridge is significantly smaller than that of wild-type littermates (Kreidberg *et al.*, 1993). Recent studies into the role of WT1 in early gonadal development indicates that WT1 regulates the expression of SF-1, a gene that it important for the development of the gonad (Wilhelm *et al.*, 2002). Analysis of SF1 null mice demonstrates that in the absence of SF-1, gonadal development does not proceed past the early bipotential progenitor stage (Luo *et al.*, 1994). Therefore, as is the case in development of the kidney, WT1 is fundamental for the early stages of gonadal development. It is required for the establishment and maintenance of the early bipotential progenitors of the gonad, possibly through the regulation of SF-1.

Renal abnormalities caused by gene knockouts are not normally lethal until the immediate postnatal period. WT1 null mice die between E13 and E15. Histological examination reveals that the hearts of the WT1 null mice are malformed and exhibit ventricular myocardium hypoplasia. The embryos are edemic and pericardial bleeding was observed (Kreidberg *et al.*, 1993). In addition to the malformation of the heart, they also exhibited incomplete diaphragmatic separation of the thoracic and abdominal cavities, and aplastic lungs.

1.7.2 YAC Complementation of the WT1 Knockout Mice

In an attempt to rescue the WT1 embryonic lethality, and to explore the later role of WT1 in development, a human WT1 YAC construct was introduced to complement the WT1 null mice (Moore *et al.*, 1999). The authors were able to show that the human WT1 YAC was able to completely rescue the heart defects and mid-gestation lethality, with the null mice rescued until birth. From this, the authors concluded that WT1 was crucial for correct epicardial development.

Recent work has extended these findings and suggested a crucial role for WT1 in the development of the epicardium and of the heart (Moore *et al.*, 1999; Perez-Pomares *et al.*, 2002). The epicardium generates a population of epicardially derived cells (EPDCs) that invade a variety of developing heart tissues in a well-defined spatiotemporal manner (Perez-Pomares *et al.*, 2002). These EPDC progenitor cells retain the potential to differentiate into coronary smooth muscle, cardiac fibroblasts and coronary endothelium. Closer analysis of the expression of WT1 in the epicardium layer demonstrates that WT1 is expressed in both in the epicardium and the EPDC progenitor cells that delaminate from this layer (Moore *et al.*, 1999; Perez-Pomares *et al.*, 2002). Initially, WT1 is present in the undifferentiated progenitor cells as they begin their migration into the developing myocardial layer of the heart. However as they migrate deeper into the myocardial layer and undergo terminal differentiation to produce smooth muscle and endothelial cell lineages, the expression of WT1 becomes down-regulated. This down-regulation of WT1 correlates with the upregulation of lineage-specific differentiation. When the development of the epicardium is delayed there is a reduction in the number of migrating invasive WT1 positive progenitor EPDCs, resulting in abnormalities within the coronary vessels and thinning of the ventricular myocardium (Perez-Pomares *et al.*, 2002). In relation to the development of the heart in the WT1 null mice, analysis of the epicardium layer demonstrates that the epicardium develops abnormally, and there is a reduction in

the number of progenitor EPDCs (Moore *et al.*, 1999). Therefore the correct formation of the ventricular myocardium is dependent upon the invasion of WT1 positive progenitor EPDCs.

The YAC complementation experiments also pointed to an important role for WT1 in the formation of the adrenal gland (Moore *et al.*, 1999). In the analysis of the complemented WT1 YAC mice, it was noted that the adrenal gland was absent. Interestingly WT1 expression has not been reported in the adrenal gland, but is restricted to the outer mesothelial lining (Rackley *et al.*, 1993). This suggests that the WT1 might be required in the early stages of adrenal gland formation, possibly during a developmental stage when the gonad and adrenal gland originate from a common progenitor population.

The YAC rescue experiments also highlighted the continuous requirement for WT1 during nephrogenesis. The analysis of the complement YAC WT1 null mice illustrated a varying degree of kidney development ranging from complete absence of kidneys to the development of hypoplastic kidneys. However, the YAC rescue experiments only partially rescued nephrogenesis in the WT1 null background, with nephrogenesis arrested at the comma shaped bodies (Moore *et al.*, 1999). The failure to obtain complete rescue could be explained by too low an expression level of the human YAC transgene, with levels of only approximately 10% of WT1 levels being achieved (Guo *et al.*, 2002).

To further dissect the role of WT1 in nephrogenesis, the YAC complementation experiments were repeated using a human YAC that spanned 470 kb of the WT1 locus (Guo *et al.*, 2002). Using this larger YAC, and depending on the expression levels of *WT1* transgene, this construct could fully rescue the WT1 null nephrogenic phenotype. In rescued mice that possess two copies of the human WT1 YAC, and hence a higher level of WT1 expression, 75% of the transgenic mice developed two kidneys. Although born with two kidneys, the YAC rescued

transgenic mice developed glomerular sclerosis. Extending the analysis of these mice, it was demonstrated that the expression levels of podocalyxin and nephrin, two podocyte specific genes, were down-regulated in rescued YAC transgenic mice. The authors concluded that the onset of the renal disease and the reduced expression of the podocyte specific genes were a direct consequence of reduced levels of WT1 during nephrogenesis. However the possibility exists that the observed phenotype of the YAC rescued mice could be due to an inherent development defect in the kidney, caused by the inability of the human YAC to fully rescue the WT1 nephrogenic phenotype. However in conjunction with the earlier YAC experiments, it establishes that WT1 is required for the later stages of nephrogenesis.

1.7.3 The Effect of Genetic Backgrounds on the WT1 Knockout Phenotype

The original study describing the phenotype of WT1 knockout mice was carried out on a 129/C57BL/6 genetic background (Kreidberg *et al.*, 1993). The WT1 mutation has been bred onto different mixed mouse backgrounds with the effect of delaying embryonic lethality until birth (Herzer *et al.*, 1999). As with the YAC complementation studies, this system allowed the requirement of WT1 for organogenesis in the later stages of embryogenesis to be assessed. It was demonstrated that on a mixed genetic background, WT1 null mice where asplenic, implicating a crucial function for WT1 in spleen development (Herzer *et al.*, 1999). This failure of spleen development was found to correlate with an enhanced rate of apoptosis of the progenitors that form the spleen.

By breeding the WT1 null mutation on a mixed background, WT1 has been also shown to be required for normal development of the retina (Wagner *et al.*, 2002). In the developing retina, WT1 transcripts are initially detected throughout the neural retina and in the developing lens vesicle of E12 mice. Between E15 and P1, WT1 expression becomes restricted to the presumptive retinal ganglion cell layer. In the

WT1 null mice at E12, there was a reduction in the number of retinal progenitor cells. At E18 the WT1 null mice have a retinal layer that was consistently thinner than that of age-matched littermates. This reduction in the retinal layer was shown to be caused by an increase in apoptosis of retinal ganglian cells (Wanger *et al.*, 2002). Therefore, the abnormalities in the WT1 null mutant retinas indicate that WT1 is required for two stages during retinogenesis, firstly in the maintenance and proliferation of retinal progenitor cells, and then in the development of retinal ganglion cells (Wagner *et al.*, 2002).

1.7.4 +/- KTS Knockout Mouse

Considerable evidence suggests that the WT1 KTS isoforms have different biochemical functions, however what are their properties *in vivo*? Recently, by gene targeting, mouse models have been generated which express either WT1 (+KTS) or the WT1 (–KTS) isforms in isolation (Hammes *et al.*, 2001). To ablate the +KTS specific isoforms, a mutation was introduced into the second splice donor site, mimicking a mutation found a human patient with Frasier syndrome (-KTS isoforms only generated, Frasier mice). The –KTS specific isoforms were ablated by destroying the first splice donor site (+KTS isoforms only generated, KTS ablation mice). These models allow the *in vivo* properties of the KTS isoforms to be analysed.

The development of the heterozygote Frasier and KTS ablation mice was normal, and adults were fertile producing normal numbers of offspring. However after a period of 2-3 months, a proportion of the Frasier mice (70%) presented with glomerular sclerosis and died due to renal insufficiency. This phenotype mimicks what has recently been observed in the WT1 heterozygous null mice, but at an accelerated rate (Menke *et al.*, in press).

In both Frasier and KTS ablation mice, homozygous mice survived until birth, but then died 24 hours after birth. Analysis of kidney development demonstrated that although there were differences in the gross development of the kidney, in contrast to the WT1 null mice, nephrogenic induction was initiated and both mouse models demonstrated presence of glomeruli. Histological analysis of the Frasier (-KTS isoforms only) kidneys demonstrated that there was an increase in the stromal tissue of the kidney and a decrease in nephrogenic zone, although the number of glomeruli generated appeared to be normal. Closer inspection of the podocytes revealed their architecture was disrupted and foot process formation was impaired. The podocytes lacked the typical flatting of the glomerular basement membranes (GMB) and analysis of markers of the GBM, synaptopodin and α3 integrin, showed aberrant expression.

Histological analysis of the KTS ablation (+KTS isoforms only) showed a more severe phenotype in the kidney. The kidneys were small, with an increase in the stromal component and a decrease in the nephrogenic zone. The number of glomeruli was reduced, and the glomeruli that did form were small and dramatically contracted. There was no expression of synaptopodin or α3 integrin. Overall, this indicated that both WT1 KTS isoforms are required for specifically for the differentiation and formation of glomeruli.

In the analysis of the gonadal development, there were distinct phenotypes in the Frasier and KTS ablation mice. In all homozygous XY Frasier mice there was complete sex reversal, whilst in the KTS ablation mice the gonads were dramatically reduced in size and mainly consisted of undifferentiated mesenchyme, with an absence of differentiated cell types. Therefore, it would appear that –KTS isoforms maybe required for the survival of the gonadal primordium, and that the +KTS isoforms are involved in the male sex determination pathway. This requirement for –KTS isoforms in the survival of the bipotential gonadal progenitors correlates well with the regulation of SF-1 by –KTS isoforms (Wilhelm

et al., 2002). The apparent role of +KTS isoforms in male sex determination correlates with the potential regulation of *Sry* by WT1 (Hossian and Saunder, 2001). *Sry*, the mammalian testis determining gene, is responsible for the differentiation of the gonadal primordium along the male sex determination pathway. In the Frasier mice, the levels of *Sry* were reduced to a level of 25% of that of wild-type littermates.

Although the KTS models demonstrate a crucial role for the individual isoforms in the correct formation of the kidney and gonad, it would appear that in the development of the adrenal gland, heart and spleen, there is a level of redundancy with each isoform being able to perform identical functions. Also it would appear that unlike the situation in the WT1 null mice, the initial stages of nephrogenesis are also unaffected.

1.7.5 Mammalian Isoforms of WT1

Mouse knockouts of WT1, or one of the evolutionary conserved KTS isoforms, has clear implications for the role WT1 and the KTS isoforms in kidney and gonadal development (Kreidberg *et al.*, 1993; Hammes *et al.*, 2001). However, what are the functions of the mammalian specific isoforms of WT1? One suggestion is that the WT1 mammalian isoforms maybe involved in mammalian-specific developmental processes such as embryonic implantation or lactation.

To determine the function of the mammalian exon 5 containing isoforms, mouse models were generated in which these isoforms were ablated using gene targeting (Natoli *et al.*, 2002). Surprisingly mice homozygous for the absence of exon 5 developed normally, and both males and females were fertile (Natoli *et al.*, 2002). Recently a mouse model to determine the function of the mammalian-specific CTG isoforms during development has also been generated through gene targeting (Miles *et al.*, 2003). The CTG isoforms contain a 68 amino acid N-terminal

extension that arises from an upstream CTG initiator codon. Proteins derived from this alternative start site behave in a similar manner to WT1 isoforms derived from the main ATG start site; they are localised to the cell nucleus and are capable of mediating transcriptional repression in reporter assays (Bruening *et al.*, 1996 (b)). Western blot analysis of a range of tissues and cell lines shows that the CTG isoforms are present in all WT1 expressing tissues and cells lines, including ovary, uterus kidney and Wilms' tumour (Bruening *et al.*, 1996). Ablation of the CTG isoforms was achieved by introducing a translation stop sequence downstream of the CTG start site (Miles *et al.*, 2003). As with the exon 5 knockout mice, CTG null mice were observed to be viable and healthy up to one year of age. Therefore, both models indicate that the mammalian-specific WT1 isoforms are dispensable for mammalian development. Why the mammalian *WT1* gene produces numerous proteins isoforms remains unclear.

1.8 Aims and Summary of Project

There are several genomic loci associated with sporadic Wilms' tumour which have been mapped, but only the Wilms' tumour suppressor gene, *WT1*, has been cloned and shown to be directly involved in urogential development. Studies of the expression pattern, patients with *WT1* mutations, and also the generation of a WT1 knockout mouse model, have demonstrated that WT1 has a fundamental role in the development of the genitourinary system.

The *WT1* gene is an example of a gene that generates several proteins isoforms, with each isoform possessing differing biochemical properties. It serves as a paradigm for mutlifunctional proteins, in that it appears to be involved in transcriptional regulation and also post- transcriptional RNA processing

Taking into account some of the findings from the study of *WT1* gene function, it would appear that WT1 has several roles during development. Firstly, WT1 is

expressed in the developing nephrogenic and gonadal lineages. In both of these lineages, there is a mesenchymal-to-epithelial transition. On the basis of the WT1 expression in these tissues, it was proposed that WT1 may mediate this transition (Pritchard-Jones *et al.*, 1990). From the study of the WT1 knockout mouse and the YAC complementation experiments, it is clear that WT1 is crucial for this event and mediates the terminal differentiation of these two lineages. However, in contrast to the situation in the nephrogenic and gonadal lineages, mounting evidence suggests that in the other sites of WT1 expression it would appear that its function may be required to maintain cells in an undifferentiated progenitor state. In the heart, WT1 is expressed in the epicardially derived progenitor cells. As the progenitor cells undergo terminal differentiation to form the smooth muscle and endothelial cell lineages, the expression of WT1 becomes down-regulated. This down-regulation of WT1 correlates with the upregulation of lineage specific markers. In the haematopoetic system, WT1 is expressed in haematopoetic progenitors in the bone marrow, and then is down-regulated as differentiation to specific lineages occurs. And finally in the retina, WT1 expression has been detected in retinal progenitor cells and then is down-regulated upon terminal differentiation.

Although there are a number of clues as to the function of WT1, important questions remain unanswered, particularly the role of WT1 in the adult. WT1 expression in the adult is restricted to discrete pockets of expression in the sertoli cells, mesothelium and the podocytes. From studies of the WT1 heterozygous null mice, data suggests that WT1 has a role in the maintenance of normal physiology of the kidney. However the function of WT1 in the mesothelium and the sertoli cells remains unclear. In addition there are no adequate mouse model to analyse the role of WT1 in tumourgenesis.

The WT1 knockout mouse created for the molecular analysis of the role of WT1 in urogenital development resulted in embryonic lethality at midgestation. The

analysis of mutant embryos revealed a failure of kidney and gonad development, establishing a fundamental role for WT1 in early urogenital development. However the model system is limited, as it prevents insight into the role of WT1 in the later stages of embryogenesis and post-natal development. The first chapter of this thesis, Chapter 3, describes the use of gene targeting in embryonic stem cells to generate a conditional allele of *WT1* using the *Cre-LoxP* system. Combining specific promoters and inducible *Cre* recombinase- expressing transgenic mouse lines, the establishment of this conditional mouse model will provide a greater understanding of the pathways and function of WT1, in both the later stages of embryogenesis and in adulthood. It is also hoped that this strategy will produce a mouse model that will recapitulate the human pathology of Wilms' tumour and the associated urogenital abnormalities.

The best characterised function of WT1 is as a transcription factor. Reporter construct assays and over-expression of single WT1 isoforms in tumour derived cell lines have suggested putative target genes for both transcriptional activation and repression by WT1. However, significant variation has been found using these approaches and often the results from these assays do not often reflect the situation *in vivo*. The range of physiologically relevant targets that have been identified to date is relatively small, with only SF-1 and amphiregulin being confirmed with some certainty. A huge problem in the study of WT1 function and identification of targets has been the limited availability of cell lines that express endogenous WT1 at detectable levels and also the limitations of the whole animal model. Therefore a more suitable *in vitro* system may better facilitate the identification of downstream targets.

Recently, it has been shown that differentiation of embryonic stem cells with *all-trans* retinoic acid induces high level expression of WT1. Chapter 4, describes the use of retinoic acid differentiation in embryonic monolayer cultures to study WT1. From the *in vitro* differentiation of ES cells, and the use of specific lineage

markers, this study has established that WT1 is expressed in neuronal progenitors within this system. Analysis of WT1 expression *in vivo* confirms that WT1 is expressed in neuronal progenitors specifically in the ventral neural tube.

Chapter 5, describes the generation of WT1 null ES cell lines through sequential gene targeting of both alleles of *WT1*. Using retinoic acid differentiation of ES monolayers, followed by Affymetrix genechip microarray analysis, the effects of WT1 loss-of-function on gene expression is examined. From this study, a number of novel WT1 target genes are identified.

Several protein partners have been identified that either modulate the function of WT1 as a transcription factor, affect the activity of WT1 in cell cycle regulation and apoptosis, or which implicate a role for WT1 in RNA splicing and post-transcriptional regulation. Thus, Chapter 6 describes the use of the RA differentiation in ES cells followed by endogenous immunoprecipitation of WT1 and MALDI TOF mass spectrometry, to identify a novel WT1-interacting protein partner.

Chapter 2 Material and Methods

Chapter 2 Materials and Methods

2.1 DNA Manipulations

All chemicals were analytical grade and were supplied by Sigma, Promega, Gibco BRL, BDH, Fisher Scientific, Flowgen, and Roche. Nucleic acid manipulations were done in 1.5 ml centrifuge tubes unless otherwise stated. General solutions were prepared by HGU technical staff and autoclaved and stored at room temperature.

2.1.1 Preparation of Genomic DNA

Tissue samples were re-suspended in 500 µl of tail mix buffer. 5 µl of proteinase-K (10 mg/ml) was added with gentle mixing. The samples were incubated at 55°C overnight, followed by a 1hr incubation at 37°C with 10 µl of 10mg/ml DNAse-free RNAse (Roche). The DNA was cleaned by 2 x phenol/chloroform extractions, plus one chloroform extraction. DNA was precipitated from the aqueous layer with 0.6 volumes of 100 % ethanol and centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol. The supernatant was discarded and the pellet was allowed to dry at room temperature and then re-suspended in ddH_20.

2.1.2 DNA quantification

For concentrations of DNA thought to be in excess of 300 ng/µl, the sample was diluted 1:200 in ddH_20 and the absorbance was read at 260/280 nm on a spectrophotometer. For samples less than 300 ng/µl, 1-2 µl of the DNA was run on a 1% agarose/1 x TAE ethidium bromide (1 µg/ml) stained gel, alongside known amounts of standard DNA. The concentration of the sample was then estimated by comparing the intensity of its fluorescence to that of the standards under a UV light.

2.1.3 Restriction Digestions

Digestions of DNA with restriction endonucleases were carried out in the appropriate buffer at the recommended temperature. Restriction enzymes were supplied by Boehringer Mannheim and NEB. DNA digestion with restriction endonucleases were performed using the optimal conditions as recommended by the particular manufacturer. Digests were performed using 1x buffer, DNA (10 ng to 1 μg/μl) and 0.5-5 units of enzyme per μg of DNA. The total volume of enzyme was always limited to no more than 10% of the reaction volume to avoid glycerol inhibition of digestion. Incubations were at 37°C, unless otherwise instructed, for periods from 1hr to overnight.

2.1.4 DNA Electrophoresis

DNA molecules were separated according to their size by agarose gel electrophoresis.

The percentage of agarose (BioGene) used to make the gel was dependent on the size range of the DNA molecules to be separated. Digested DNA for Southern blot analysis was run on a 0.8% agarose gel, whilst PCR products were commonly run on agarose gels of between 1%- 2%. If DNA fragments were to be purified from the gel then a low melting point agarose (Flowgen) was used. The agarose gels were made in TAE buffer (20 x TAE : 0.8 M Tris, pH 8.0; 20 mM EDTA; 0.4 M acetic acid) with ethidium bromide added (0.5 μg/ml). DNA was mixed with 10 x loading buffer (20% ficoll; 100 mM EDTA; Orange G (Sigma)). A DNA ladder was used to determine the size of DNA fragments. Following electrophoresis DNA fragments were visualised on an UV trans-illuminator.

2.1.5 Southern Blot

Between 5-10 μg of genomic DNA was digested overnight with the appropriate restriction endonuclease(s). The sample was then mixed with 5 μl of orange-G loading dye, and loaded onto an ethidium bromide (0.5 μg/ml) stained agarose (0.8% 1 x TAE gel). A suitable DNA marker ladder was used to determine the size range of the position of the fragments of interest. Samples were electrophoresed until the orange-G ran off the end of the gel. The gel was then photographed with UV using a video camera and image processing system (BioRad) and the position of the DNA size markers were noted with a ruler. The gel was soaked in 0.25 M HCl for 20 minutes at room temperature, with gentle agitation. The gel was then washed twice with denaturing buffer (0.5 M NaOH/1.5 NaCl) for 20 minutes. The gel was inverted and then placed onto a piece of Saran wrap or cling film on the bench. The following was then placed on top of the gel: 1 piece of membrane (Pall) soaked in 2 x SSC, 2-4 pieces of 2 x SSC soaked 3 MM Whatman paper, followed by a 5 cm stack of dry paper towels, a glass plate and two 500 ml bottles half filled with water (weight approximately 0.5 kg). The blotting apparatus was left overnight. The apparatus was dismantled and the position of the wells marked on the membrane with a pencil. The membrane was then UV cross-linked.

2.1.5.1 Labelling of DNA probes

The labelling reaction was carried out using the Random Prime kit (Boehringer Mannheim). 100 ng of the DNA probe to be used in the Southern blot was re-suspended in a total volume of 10 μl of ddH_20 and denatured for 5 minutes at 100°C and then transferred to ice. To this 5 μl of reaction mix (1μl dATP, 1μl dTTP, 1 μl dGTP and 2 μl reaction mix), 4 μl of 10μCi/μl ^{32}P dCTP and 1 μl of klenow (2U/μl). The reactions were incubated at 37°C for 1 hour. The labelled

DNA was separated from the unincorporated nucleotides by spinning the labelling reaction through TE equilibrated spin columns (Amersham Pharmacia).

2.1.5.2 Hybridisation and Washes

Hybridisation and washing of Southern blots was carried out in roller bottles in a hybridisation oven at 65°C overnight. Hybridisation of probes to the blots was carried out in Church and Gilberts solution (0.5 M Na_2HPO_4 (pH7.2), 20%SDS, 0.5 M EDTA). DNA probes were boiled for 5 minutes and added to the hybridisation solution and incubated overnight. The following day, the blot was washed 2 x 30 minutes in 2 x SCC/0/1% SDS, 1 x 30 minutes in 1 x SSC/0.1% SDS, and a final 1 x 30 minute wash in 0.1 SSC/0.5 SDS. The membrane was then wrapped in Saran Wrap and exposed a Molecular Dynamics Phosphoimager screen. The image was then scanned into the phosphoimager and analysed using the ImageQuant software package.

2.1.6 PCR

For PCR reactions, filtered Gilson tips were used in each assay. dNTPs were purchased as stocks of 100 mM (Abgene). Working stocks of 10 mM were made by mixing 10µl of each of the dNTPs (dATP, dCTP, dGTP, dTTP) with 60 µl ddH_2O to a final volume of 100 µl. Stocks were stored at –20°C. dNTPs were used in PCRs at a final concentration of 0.2 mM. Primers were purchased from MWG Biotech as lyophilised desalted compounds. Stocks were made up to 1 µg/µl using sterile dH_2O. Primers were used in PCRs at a final concentration of 100 ng/µl. Ampli*Taq* (5U/µl) was used at 0.2 µl per 25 µl reaction (Applied Biosystems). PCR buffer was a 10X stock and therefore diluted 1:10 for reactions (Applied Biosystems). Mg^{2+} was used at a final concentration of 1.5 mM (Applied Biosystems). Routine PCRs were done in a MJ Research DNA Engine Tetrad. 100-200 ng of genomic DNA was used for PCR reactions and generally all PCR amplification programmes were identical (35 cycles). The

exception was the annealing temperature, which was varied according to the primers used:

General PCR amplification programme:

1. 94°C for 5 mins
2. 94°C for 45 secs
3. n°C for 60 secs (variable annealing temperature)
4. 72°C for 1 min
5. 72°C for 5 mins

2.2. Microbiology

Aseptic technique was observed for all steps involving the growth of bacterial cells (setting up cultures, pouring agar plates, selecting single colonies, and storing bacterial stocks). Liquid cultures were grown at 37°C with vigorous shaking and dry cultures were grown on inverted agar plates at 37°C. HGU technical staff prepared all bacterial growth media stocks.

2.2.1 Production of electrocompetent cells

A single colony of XL1 Blue *E.coli* cells from an agar plate was used to inoculate approximately 10 ml L-broth for overnight growth. The culture was used to inoculate 2 x 400 ml fresh L-broth the following morning. Cells were grown to an absorbance at 600 nm (A_{600}) of 0.15-1.0. Flasks were chilled on ice for 15-30 minutes and cells were centrifuged at 4°C, 4000g for 15 minutes. Pellets were re-suspended in 800 µl ice cold sterile 10% glycerol. The centrifugation step was repeated and cells were re-suspended in 400 ml 10% cold glycerol. Following a further centrifugation step, cells were re-suspended in 20 ml 10% glycerol. Cells were centrifuged once again and re-suspended in 2-3 ml 10% glycerol. The final concentration of cells was approximately $3x10^{10}$ cells/ml. Aliquots of cells were frozen in liquid nitrogen and stored at –70°C.

2.2.2 Transformations

Competent cells were transformed with DNA by electroporation. Electrocompetent cells were thawed on ice and 50 µl were added to each DNA sample. The mixture was transferred to ice cold electroporation cuvettes and allowed to sit for 1 minute on ice. Cells were electroporated using a BioRad electroporator set at 25µF, 2.5kV, and 200Ω. 1 ml L-broth/ Mg^{2+} was added to the cells immediately following electroporation and cells allowed to recover for 1 hour by shaking at 37°C prior to plating on selective medium.

2.2.3 Isolation of plasmid DNA

Plasmid DNA was prepared using commercially available kits (Qiagen). For the extraction of small amounts of plasmid DNA (Qiagen Plasmid Mini Kit), a single colony was used to inoculate approximately 10 ml L-broth with antibiotic selection (ampicillin) for overnight growth. The following morning, plasmid DNA was extracted using the kit according to the manufacturer's instructions. Plasmid DNA was eluted in 30 or 50 µl elution buffer. For large amounts of plasmid DNA, single colonies were grown as described above and large cultures of L-Broth (200-350 ml) with antibiotic selection were inoculated with 500 µl from the 10 ml overnight cultures. The following morning, plasmid DNA was extracted using the kit (Qiagen Plasmid Maxi Kit) according to the manufacturer's instructions.

2.3 Mouse Embryonic Stem Cell Culture

During all procedures in ES cell culture, sterile technique was observed with use of disposable sterile pipettes (Costar). All solutions were pre-warmed to 37°C before use.

2.3.1 Maintenance of ES Cells

The ES cell line, E14.1, was cultured in BHK-21 medium (Glasgow MEM) (Gibco) supplemented with 10% FCS (Globalpharm), 0.3% glutamine (Gibco), 0.1 mM MEM non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma), 0.1 mM β-mercapthoethanol (Sigma). The culture medium was also supplemented with Leukaemia Inhibitory Factor (LIF) to maintain ES cell in their undifferentiated state. LIF was obtained from conditioned medium derived from Cos-7 cell, transfected with a LIF expression construct. The correct concentration of LIF conditioned medium added to the ES cell culture medium at any given time was derived from results of titration experiments to determine the highest dilution of LIF which supported the ES cells in undifferentiated state.

ES cells were maintained on gelatinised plates. Plates or flasks (Costar) were coated with 0.1% gelatin solution (Sigma) approximately 10-15 minutes before plating. Prior to plating of cells, the gelatin solution was removed and the ES cells were added to the flask or well in culture medium. ES cells were incubated at 37°C in 5% CO_2.

2.3.2 Gene Targeting in ES Cells

The targeting vector (*pWT1LoxP24* or *pKreWT1*) was isolated from the plasmid backbone using the appropriate restriction enzymes, gel purified using agarase and phenol:chloroform extracted. Following phenol:chloroform extraction, the targeting vector was precipitated with 100 % ethanol, washed in 70% ethanol and re-suspended to a concentration of 1μg/μl in sterile ddH_20. 100 μg of targeting vector was electroporated into the ES cell line, E14.1. $1x10^7$ ES cells were electroporated using a BioRad Gene Pulser and the setting used were 3μF and 0.8 kV.

2.3.3 Selection and Freezing of ES Clones

Following selection using G-418 (GibcoBRL)(final concentration of 250 μg/ml) or Gancyclovir (Roche) (final concentration of 2 μM) or a combination of both, surviving clones were picked. The media was aspirated from the plates, and the plates washed twice in PBS. Individual clones were isolated and placed into 96 well "V" plates with trypsin to dissociate the clone. Following 5 minutes at 37°C, the dissociated clone was transferred into a flat bottomed 96 well plate, and normal culture medium was added. Individual ES clones were cultured for a further 2-3 days. Once the clones has grown sufficiently, they were passaged into 24 wells and then split into two further wells, one well for freezing and other to harvest genomic DNA.

2.3.4 Transient Expression of *Cre*

Correctly targeted ES clones carrying the full targeting vector, *pWT1LoxP24*, were identified and expanded to carry out the transient expression of *Cre* recombinase. This step removes the *loxP* flanked selectable cassette, and generates ES clones with *loxP* sites flanking exon 1 of *WT1*. The *Cre* plasmid used was *pMC-Cre* (Gift Dr K Rajewsky). Fully targeted ES cells were grown to a final concentration of 1×10^7 in preparation for electroporation with the *pMC-Cre* plasmid. 80 μg of circular *pMC-Cre* was electroporated into ES cells containing the full targeting vector. Following electroporation, transfected ES cells were seeded at a concentration of 1×10^4 in $9cm^2$ gelatinised dishes, in normal LIF containing medium. The cells were grown in normal medium for further 4 days. Following 5 days, ES cells were placed into selection with GANC for a further 6-8 days. After 6-8 days, when clones were visible, between 30-50 clones were picked and analysed by Southern blot for the *Cre* mediated recombination. The efficiency of *Cre* recombination and GANC selection was established by a control transfection in which ES clones containing the full targeting vector were electroporated in the absence *pMC-Cre.*

2.3.5 Blastocyst Injections and Chimera Breeding

ES clones that contained *loxP* sites flanking exon 1 of WT1 were thawed into 24 wells. Clones were cultured until they reached 50-70% confluentency in normal ES medium. On the day of injection, ES clones were washed 3 x PBS and trypsinised to form single cell suspensions. The blastocyst injections were carried out by Dr. Sheila Webb. Briefly female donor mice (C57BL/6) were set up for natural matings. The following morning they were checked for vaginal plugs, and positive plugs were considered as being 0.5 dpc. Female mice were sacrificed at 3.5 dpc by cervical dislocation and the blastocysts were harvested. Typically, 10-15 ES cells were injected per blastocyst. Injected blastocysts were collected and transferred to pseudopregnant recipient female mice.

Chimeric mice generated from the injection of $WT1^{LoxP}$ ES clones were set up for germline test breeding with C57BL/6 female mice. Agouti offspring denotes contribution of the injected ES cells to the germline. Agouti mice were tailtipped and genotyped by PCR or Southern blot

2.3.6 Differentiation of Monolayer ES Cells with *all-trans* Retinoic Acid

ES cells were seeded at a defined concentration (between 1 x 10^3 x 10^4 depending up the scale of the experiment) onto 9cm^2 gelatinisized plates in normal LIF containing media. 24 hours later the cells were washed 3 x PBS and the media changed to ES culture media lacking LIF and supplemented with 1 μM *all-trans* Retinoic Acid (Sigma). At 24 hour intervals the cells were washed 3 x PBS and the media changed to fresh ES medium lacking LIF and supplemented with RA.

2.4 Protein Work

2.4.1 Preparation of Nuclear Extract and Immunoprecipitations

ES cells were seeded at 1 x 10^4 in $9cm^2$ plates. For a typical IP experiment, 20 x $9cm^2$ plates ES cells were set up. ES cells were differentiated with 1 μM *all-trans* retinoic acid as described in section 2.3.6. Following 96 hours, cells were washed 2 x with ice cold PBS and cells were scraped off into residual PBS. Cells were collected from all plates and pooled into eppendorfs. Samples are then centrifuged for 3 minutes at 4000 rpm at 4°C. The supernatant was removed and the pellet was re-suspended in 600 μl of hyptonic buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, Protease inhibitor cocktail). The samples are left on ice for 10 minutes. Following incubation on ice, the samples were vortexed and centrifuged for 1 minute at 13,000 rpm at 4°C. The supernatant was removed (cytoplasmic extract) and the nuclear pellet was re-suspended in an equal volume of lysis buffer (20 mM Hepes pH 7.9, 600 mM KCl, 0.2 mM EDTA, 20U/ml DNase, 10U/ml RNase inhibitor, Protease Inhibitor Cocktail). The samples were left on ice for 30 minutes, with samples vortexed every 10 minutes. Following 30 minutes, the samples were centrifuged for 10 minutes at 13,000 rpm at 4°C. The supernatant was collected (nuclear protein) and stored at –20°C.

The prepared nuclear extract was diluted _ with IP buffer (20 mM Hepes pH 7.5, 70 mM KCl, 5mM $MgCl_2$, 0.05% NP40, 12% Glycerol, 0.1 mM $ZnCl_2$, 1mg/ml BSA). Nuclear Extracts were firstly pre-cleared with Protein A glass beads (Prosep), to remove reduce non-specific interactions with the sepharose A. Pre-clearing was carried for 3 hours, rotating at 4°C. Beads were then washed 6 x 1 ml IP Buffer and the pre-cleared extract removed from the beads. Separately, the various antibodies were bound to the beads by adding the specific antibody to pre-washed beads (45 μl of C19 (Santa-Cruz), 9 μg of IgG and 1μl of 3G6) in 1 ml of IP buffer and rotating for 3 hours at 4°C. The pre-cleared nuclear extract was then divided for the various IPs to be carried out (WT1, hnRNP U and

Rabbit IgG control). The nuclear extract was then added to the different bound antibodies and the IP carried out overnight, rotating, at 4°C. The following day the beads were pelleted by centrifugation at 2,000 rpm and the supernatant was removed (unbound fraction). The pellet was then washed 6 x 1 ml of IP buffer. The proteins were eluted from the beads by washing the beads 3 x 50 µl aliquots of elution buffer (200 mM Glycine pH 2.5 and 1 mg/ml BSA) with agitation. Each elution removed from beads, was neutralised with 1/10 volume of 1 M Tris pH 7.5.

2.4.2 SDS-PAGE Electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was used to resolve proteins according to their molecular weight. Routinely a 10% resolving gel was used (30% acrylamide mix, 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulphate, TEMED), with a 5% stacking gel (30% acrylamide mix, 1.0 M Tris (pH 6.8), 10% SDS, 10% ammonium persulphate, TEMED).

The gel apparatus was assembled and the samples, mixed with 2 x sample buffer (62.5 mM TrisCl pH 6.8, 2% SDS, 2% β-mercapthoethanol, 10% glycerol, 0.1% bromophenol blue), were boiled for 5 minutes and loaded into the wells. Running buffer (25 mM Tris, 250 mM glycine pH 8.3 and 0.1% SDS) was added and the gel run between 150-200V. The gel was then stained with with Coomassie blue (0.1% Coomassie blue dye, 50% methanol, 10% acetic acid) to visualise proteins or transferred to nitrocellulose for Western blot analysis.

2.4.3 Western Blotting

A semi-dry blotter was used to transfer proteins onto a Hybond filter. The samples were set up on the anode plate as follows: 1 section of Whatmann filter cut to the size of the gel soaked in transfer buffer (0.3 M Tris, 10% methanol, pH 10.4). Next the Hybond filter cut to the same size as the gel was firstly soaked in 100% methanol and then equilibrated in transfer buffer. This was placed on top

of the Whatmann filter. Then the SDS-PAGE gel was soaked in transfer buffer and the carefully placed onto the Hybond filter ensuring that air bubbles were removed from the interface between the gel and the filter. Finally a section of Whatmann filter cut to the size of the gel soaked in transfer buffer was placed on top. The cathode plate was then put in place and the transfer carried out at 10V for 30 minutes.

Once the transfer had taken place, the nitrocellulose probed for the protein of interest. Firstly the nitrocellulose filter was blocked in 5% fat free milk in TBST (50 mM Tris –Hcl pH7.5, 150 mM Nacl, 0.1% Tween) for 1hour at room temperature or overnight at 4°C. The primary antibody was added for either 1hour at room temperature or overnight at 4°C. Following incubation with the primary antibody, unbound antibody was then removed by 3 x 15 minute washes in TBST. After completion of the washes, the secondary antibody conjugated with horseradish peroxidase was then added for 1 hour at room temperature. Following the incubation, the blot was washed 3 x 15 minutes with TBST. Detection of the protein was carried out using the ECL-Plus (Amersham) as described by the manufacturer.

If the filter was to be re-used for detection of another protein, the filter was stripped. This was carried out by exposing the filter to stripping buffer (100 mM β-mercapthoethanol, 2% SDS, 62.5 mM Tris.HCl pH 6.7) and incubated at 50°C for 30 minutes. The filter was then washed 2 x 10 minutes in TBST at room temperature. Detection of the protein of interest was then carried as described above.

2.5 RNA Manipulation

2.5.1 Preparation of RNA

Total RNA was isolated from embryos and ES cells using the RNAgents® Total RNA Isolation System (Promega) following the manufactures instructions.

Briefly, cells or tissues were homogenised in denaturing solution (Promega). To the samples, 2M sodium Acetate (pH4.0) was added and the tubes mixed thoroughly. Tubes were then incubated on ice for 20 minutes. Following incubation, an equal volume of Phenol:Chloroform:Isoamyl Alcohol was to the tubes. The samples were then incubated on ice for 20 minutes. The samples were mixed by inversion and centrifuged for 20 minutes at 4°C. The aqueous phase was removed containing the RNA and transferred to a fresh eppendorf. An equal volume of Isopropanol was added and samples stored overnight at –20°C. To precipitate the RNA, the sample was centrifuged for 30 minutes at 4°C. The supernatant was removed and the pellet washed with 80% ethanol. The RNA pellet was then dried and re-suspended in nuclease free ddH_20. RNA was then stored at –70°C.

2.5.2 cDNA Synthesis and RT-PCR

1-5 μg of total RNA from ES cells or tissues, in 5 μl of ddH_20, was denatured for 5 minutes at 65°C and then placed on ice. To this 4 μl of 5 x reaction buffer, 1.25 μl of DNTP mix, 0.25 μl of RNA inhibitor (Roche), 1 μl of RT AMV (Roche) and 1 μl of oligo dT were added and made up to 20 μl with ddH_2O. The mix was incubated at 42°C for 1 hour. 30 μl of 1mM Tris pH 7.5 was then added and the resulting cDNA stored at –20°C until required. RT-PCR was carried out as described in section 2.1.6. Annealing temperatures for each RT-PCR was optimised for every primer pair and the cycle number ranged between 24-35 cycles depending upon the abundance of the transcript. For example, control PCRs (β-actin) were typically performed with 25 cycles, whilst 35 cycles were used to detect rare transcripts (T-brachury, MyoD)

2.5.3 Northern Blot Analysis

2.5.3.1 Preparation of Gels for Northern Blots

10 x MOPS (1 Litre use depc H_2O)
41.2 g MOPS
10.9 g Sodium acetate, 3-hydrate

3.7 g EDTA, sodium salt
Filter sterilise

17.5 mls of formaldehyde, (deionzed using mixed bed resin (Sigma)) and 30 mls 10 x MOPS was preheated to 50°C. 3-4.5 g of agarose was dissolved in 250 ml of nuclease free H_2O and allow to cool to 55°C. The pre-warmed formaldehyde, MOPS and 0.5 μg/ml ethidium bromide were added, mixed, poured immediately and the gel allowed to set. Total RNA samples were prepared using the RNAgents® Total RNA Isolation System (Promega) as per the manufactorers instructions. Individial RNA samples were prepared as below for loading:-

RNA	10μg in 8μl H2O
Deionized formaldehyde	5.5μl
Deionised formamide (Sigma)	15μl
10x MOPS buffer	1.5μl

The samples were placed at 70°C for 15 minutes to denature, the loading buffer mix was added and the samples loaded onto the gel. 8 μg RNA millenium markers (Ambion) were loaded. 1 x MOPS was used as running buffer. Following electrophoresis, the gel was visualised under UV, and the markers were marked on the gel using a needle to prick the gel. The gel was then washed in H_2O with agitation, for 15 minutes. The gel was then equilibrated in 10 x SSC for 2 x 15 minutes.

Hybond-N (Amersham) was cut to the appropriate size, wet in H_2O then equilibrated in 10 x SSC transfer buffer. A tray was half filled with transfer buffer, a platform was placed in the tray and the platform was covered with a wick made from 3 pieces of 3 MM paper soaked in transfer buffer. The gel was placed upside down on the wick so that the bottom of the gel was facing up. The gel was then surrounded with parafilm to avoid transfer buffer being absorbed on to the paper towels. The membrane (Hybond-N) was placed on top of the gel and rolled out with a clean pipette to remove any trapped air bubbles. Three sheets of 3 MM paper cut to size the size of the gel and saturated in transfer buffer, were

placed on top of the membrane. Again a clean pipette was to used to roll out the paper to avoid trapping any air bubbles. On top of the 3 MM paper, a stack of paper towels were placed. A glass plate and a weight not exceeding 750 g was placed on top of the stack of paper towels. The blot was left overnight to transfer. The following day, the blotting apparatus was dismantled. Before separating the gel and membrane, both were inverted and wells from the gel were marked on the membrane with a pencil. The markers placed on the gel for the marker lane were also marked on the membrane. The membrane was then UV cross-linked.

2.5.3.2 Hybridisations and Washes

For hybridisations, Ultrahyb hybridisation buffer (Ambion) was used. The Ultrahyb solution was warmed to 60°C to completely dissolve. The membrane was placed into the Ultrahyb buffer and pre-hybridised for 3 hours at 48°C. Following pre-hybridisation, the labelled probe was added to the membranes and hybridised overnight. The following day, the membranes was washed at 48°C in 2 x SSC/0.1% SDS for 30 minutes. The membranes were then washed at 65°C with the following washes:- 1 x 30 minute wash with 1 x SSC/0.1% SDS, 1 x 20 minute wash with 0.2 x SSC/0.1%SDS and finally 1 x 15 minute was with 0.1 x SSC/0.1% SDS. The membrane was then wrapped in Saran Wrap and exposed a Molecular Dynamics Phosphoimager screen. The image was then scanned into the phosphoimager and analysed using the ImageQuant software package.

2.6 Affymetrix Gene Chip Arrays

ES cells were harvested at the various time points and RNA was collected using the RNAgents® Total RNA Isolation System (Promega) according to the manufacturers recommendations. The Affymetrix Mouse U74Av2 GeneChips® were used for analysis of gene expression (Affymetrix). Hybridisations and analysis of the arrays was carried out by Dr. Kevin Robertson from the GTI at the University of Edinburgh. RNA quality was checked using a Bioanalyser (Agilent Technologies). Double stranded cDNA was prepared using the Custom

SuperScript ds-cDNA kit (Invitrogen) and biotin-labelled cRNA complex probes prepared using the BioArray High Yield kit (Enzo, New York, US) according to manufacturers recommendations. The cRNAs were fragmented and hybridised to Affymetrix Mouse U74Av2 GeneChips® according to Affymetrix protocols (Affymetrix). Data from all chips was analysed using Affymetrix Microarray Suite (version 5.0).

2.7 MALDI-TOF

2.7.1 SDS-Polyacrylamide Gel Electrophoresis

20 μg WT1 immunoprecipitates were mixed with 20 μl of sample buffer containing Tris-HCl (0.06M), Glycerol (10% v/v), SDS (2% w/v), 2-β-mercaptoethanol (5% v/v), Bromophenol blue (0.025% w/v) and heated to 100°C for 5 min. Electrophoresis was performed on 20μl of each sample in a NuPAGE™ 4-12% Bis-Tris Gel (Invitrogen) at 200 Volts for 40 min. Mark12 Unstained Standard marker (Invitrogen) was used to estimate molecular weight within the range 200 kDa to 2.5 kDa and protein bands were visualised with Colloidal Coomassie Blue Stain (Genomic Solutions).

2.7.2 In-Gel Tryptic Protein Digest

MALDI-ToF analysis was carried out by Mr. Nick Tomczyk from the Chemistry Department at the University of Edinburgh. After visualisation, protein bands of interest were excised and de-stained with 50% acetonitrile until all Coomassie blue was removed from gel slice. Protein bands were digested using a protocol modified from Steen *et al* (Steen *et al.*, 2002). Resultant peptides were desalted with C18 ZipTips (Millipore) ready for MALDI-ToF analysis.

2.7.3 Mass Spectrometric Analysis

Peptide fingerprint analysis was carried out using an Applied Biosystems Voyager DE™ STR, MALDI -TOF instrument. Samples (0.5 μl) were co-crystallized with 0.5 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, directly onto MALDI plate. Interpretation of data was performed with the "Voyager V5 Data Explorer" software and database searching performed using the Protein Prospector MS-Fit program on the www at the University of California at San Francisco (http://prospector.ucsf.edu/ucsfhtml4.0u/msfit.htm).

2.8 Immunohistochemistry

2.8.1 Immunofluorescence on ES Cells

Undifferentiated ES were seed onto gelatinised slides placed in $9cm^2$ petri dishes. ES cells were then differentiated following the method described in 2.3.6. After a set period of time (0-96 hours), the slides were carefully washed 3 x PBS, and ES cells were fixed in 2% PFA (PFA dissolved in PBS) for 10 minutes at 4°C. Slides were then incubated in blocking buffer (10% FCS in TBST (50 mM Tris-Hcl pH7.5, 150 mM NaCl, 0.1% Triton X-100)) overnight at 4°C. The following day primary antibodies were added at the required concentration in blocking buffer, and incubated at room temperature for 1 hour. The slides were then washed 3 x 5 minutes with TBST, and the secondary antibody (Alexa Fluor 594 and Alexa Fluor 488; Molecular Probes) was added at a concentration of 1:400 and left at room temperature for 1 hour. Finally the slides were then washed 3 x 5 minutes with TBST, mounted (using vectastain containing DAPI) and visualised using a Zeiss microscope.

2.8.2 Immunofluorescence of Tissue Sections

E9.5 and E10.5 embryos were dissected out and fixed in 4% PFA at 4°C for 1hour. The embryos were then transferred to 30% sucrose in PBS overnight. Embryos were then transferred to 30% sucrose in PBS and left overnight at 4°C.

The following day, embryos were transferred to O.C.T.™ (Sakura), positioned to the plane of section required and frozen at –70°C. 10 μM section were cut using a cryostat and placed onto slides (Superfrost, BDH)

Sections were then blocked overnight in blocking buffer (10% FCS in TBST (50 mM Tris-Hcl pH7.5, 150 mM NaCl, 0.1% Triton X-100 0.1% Nazide)) at 4oC. The following day, sections were incubated at room temperature with the required primary antibody for 6-8 hours. Sections were then washed 3 x 15 minutes in TBST. The secondary antibodies antibody (Alexa Fluor 594 and Alexa Fluor 488; Molecular Probes) in blocking buffer, were then added to the sections overnight at 4°C. The next day, sections were washed 3 x 15 minutes in TBST, and mounted in vectashield mount. The sections were visualised using Zeiss microscope.

Chapter 3 Generation of a Conditional Allele of *WT1*

Chapter 3 Generation of Conditional Allele of *WT1*

3.1 Introduction

3.1.1 Gene Targeting Strategies in ES Cells

Over the last decade, the use of gene targeting by homologous recombination in ES cells for the production of mouse knockout mutants, has provided important discoveries in the field of genetics and development biology (For review see Capecchi, 2001). Strategies for gene targeting are based around the use of two basic types of vectors, sequence replacement or sequence insertion vectors, which differ in their mechanisms of chromosomal integration and gene targeting frequencies.

Replacement vectors are most frequently used for gene targeting in ES cells and are usually employed to inactivate the function of the gene. A typical replacement vector consists of two regions of DNA, homologous to the genomic target locus (arms of homology), which are interrupted by a positive selection cassette (Thomas *et al.*, 1986). The commonly used positive selection cassettes are the bacterial aminoglycoside phosphotransferase (*neo*) gene, which confers resistance to the neomycin analogue G418 or the hygromycin B phosphotransferase (*hyg*) gene which confers resistance to hygromycin. The sequences in the 5' and 3' arms of homology, act as substrates for the double reciprocal crossover or gene conversion mechanisms that transfer the drug selection cassette into the endogenous targeted locus (Figure 3.1 (a)).

A widely used method for enriching correct homologous targeting by replacement vectors is to use a strategy that incorporates a positive/negative drug selection cassette (Mansour *et al.*, 1988). As with the vector described above, a selectable marker is included between the 5' and 3' arms of homology which is used for positive selection. Also included in the targeting vector is a

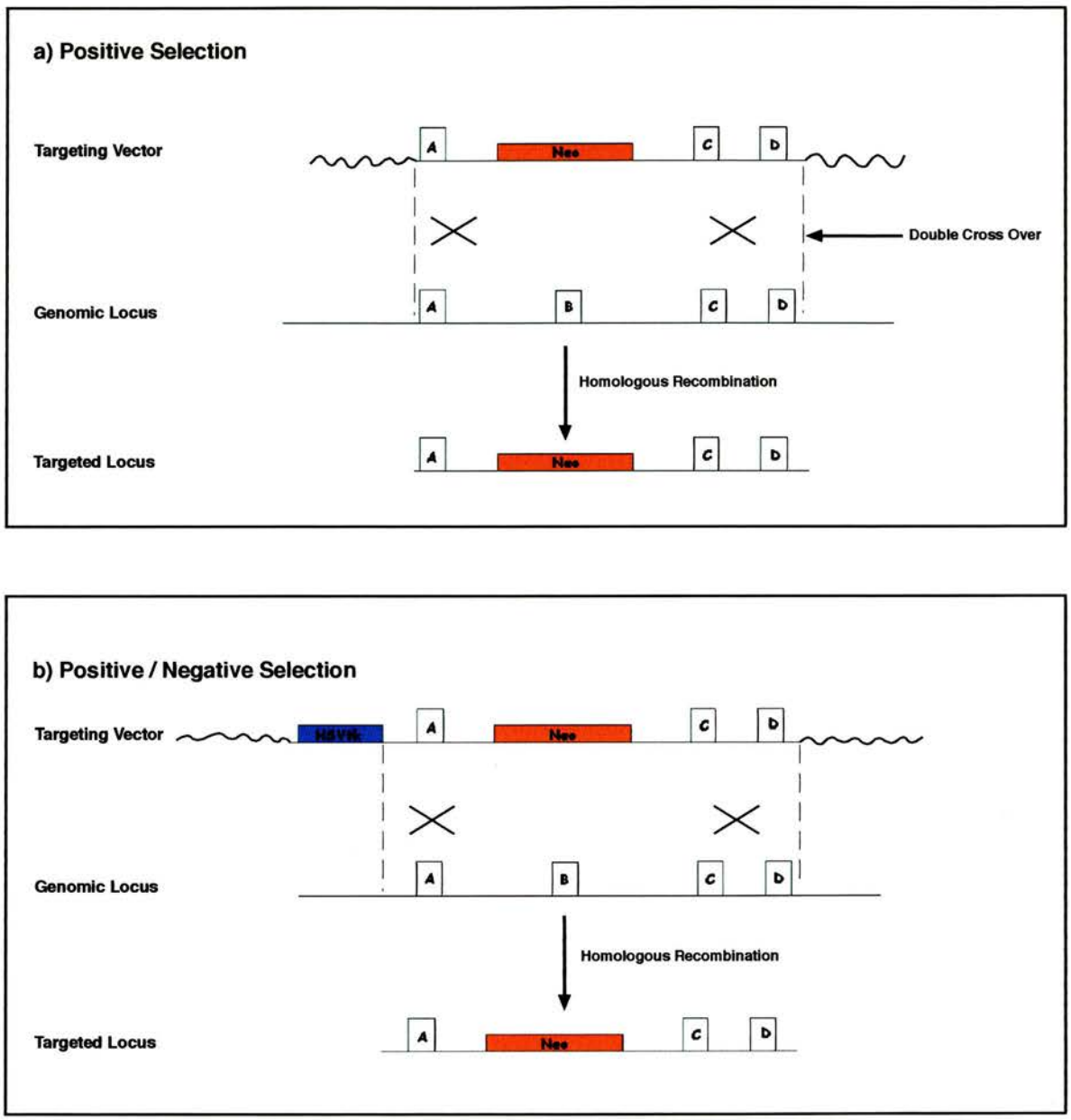


Figure 3.1 Gene Targeting in ES Cells using Sequence Replacement Vectors

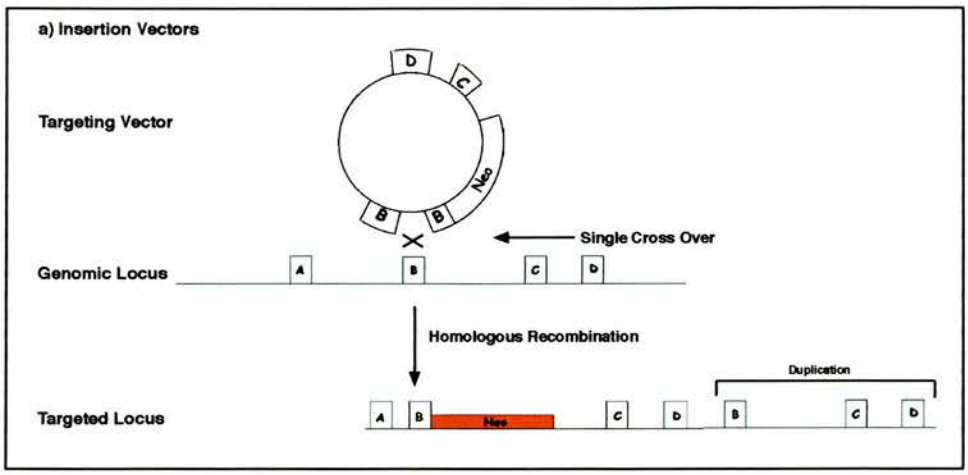
a) Sequence replacement vector for homologous recombination using positive selection. The targeting vector contains arms of homology which are interupted by a positive selection cassette, in this example, the neomycin expression cassette. Cross over points for homologous recombination are depicted by an X.

b) Sequence replacement vector for homologous recombination using positive/negative selection. The targeting vector includes the same features as above, however a negative selection cassette, the HSVtk gene, is also included. Following homologous recombination the positive selection cassette is incorporated into the target locus and the negative selection cassette is lost.

drug selection cassette for negative selection that is located at the end of either the 5' or 3' arms of homology (Figure 3.1 (b)). The most commonly used negative selection cassette is the herpes simplex virus thymidine kinase (*HSVtk*) gene. This confers sensitivity to the guanosine analogue, gancyclovir (GANC). Typically a replacement vector will randomly integrate into the genome of the majority of electroporated ES cells. These cells will retain resistance to positive selection with G148 and sensitivity to GANC. However in ES cells in which the replacement vector has integrated correctly to the targeted endogenous site, the positive selectable cassette will be incorporated into the locus, whilst the negative selection cassette (*HSVtk*) will be lost. Therefore the growth of electroporated ES cells in both G148 and GANC will enrich for the correct homologous targeting event, as randomly integrated ES clones, with an active *HSVtk* gene, will not survive the selection with GANC (Figure 3.1 (b)).

In contrast to the mechanisms of replacement vectors which undergo homologous integration by double reciprocal cross-over, insertion vectors undergo homologous recombination via a single cross-over, initiated by linear ends within a region of homology. Targeting vectors are designed to include a contiguous stretch of homologous gene sequences and a positive selection marker cassette that is included to disrupt the target gene. Insertion vectors are linearised at a point within the homology region and the linear ends acts as a substrate for chromosomal integration of the vector. Due to the insertion vectors being incorporated into the endogenous locus via a single crossover site, frequencies of homologous recombination are higher than that for replacement vectors. The frequency of targeting with insertion vectors is 5-10 fold higher than replacement vectors using the same homologous sequences (Hasty *et al.*, 1991) (Figure 3.2 (a)).

The use of insertion vectors allows for the introduction of more subtle genetic alterations to a target genomic locus. Often the presence of a neomycin gene within a target locus has been shown to adversely affect gene expression within



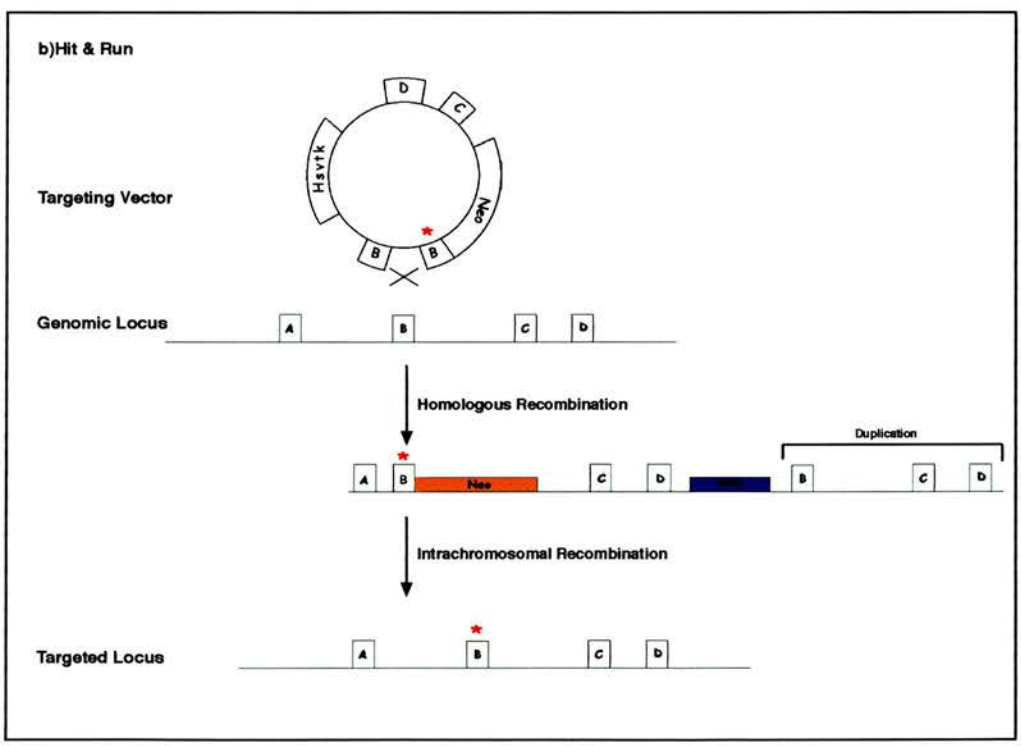


Figure 3.2 Gene Targeting in ES Cells using Sequence Insertion Vectors

a) An example of a sequence insertion vector used for homologous recombination. Homologous recombination occurs at a single cross over event (marked by an X)

b) Strategy for Hit and Run targeting in ES cells. A sequence insertion vector is introduced into ES cells carrying a specific mutation. This sequence insertion vector includes a positive and negative selection cassette. Following homologous recombination, a second selection process using GANC, is carried out for an intrachromosal recombination event, which results in the excersion of vector sequence.

the targeted locus. This was exemplified by the replacement of a hypersensitive site in the beta globin locus control region (LCR) with a neomycin selectable cassette (Fiering *et al.*, 1995). The insertion of the neomycin cassette into this hypersensitive region resulted in a 2-5 fold reduction in expression of all of the genes in the beta globin locus. In mice derived from these ES cells, which were homozygous for the deletion/replacement mutation, this caused embryonic lethality. In removing the neomycin cassette by *FLP* site-specific recombinase in ES cells, mice derived from these cells demonstrated nearly full expression of all the beta globin genes on the mutated chromosome. Therefore the presence of the neomycin cassette at the beta globin LCR region adversely affected the expression levels of genes within the targeted locus.

To overcome this problem, insertion targeting vectors can be used in a gene-targeting strategy called "hit and run". The insertion vector is composed of a genomic sequence homologous to the gene of interest, a positive selectable marker gene and a negative selectable marker gene. Following the introduction of the targeting vector into ES cells and positive selection, correct homologous recombination leads to the complete integration of the vector into the target locus. This results in a partial duplication interrupted by plasmid sequences and selection markers (positive and negative) within the target genomic locus. Next the homologously targeted ES cells are exposed to negative selection and reselected. Only clones that undergo an intrachromosomal homologous recombination event, which occurs spontaneously at a low rate in ES cells, can survive the selection procedure. These clones lose the heterologous plasmid sequences, including the selection cassettes, during this recombination event and what remains is a correctly targeted ES clone with a desired point mutation or modification in the target gene (Figure 3.2 (b)).

3.1.2 Conditional Knockouts

Classical gene targeting described above normally involves the insertion of a heterologous sequence of DNA encoding for a selection cassette (*neo* or *hyg*) into the endogenous locus, disrupting the function of the allele. This strategy is the most appropriate for testing the function of a gene that is involved in development or involved in particular biological functions. However this approach has its limitations if one is faced with a gene whose ablation results in embryonic lethality. Although pointing to a crucial role for this gene during embryogenesis it renders the mouse model useless for the study of the loss of function of that gene in later developmental processes and in adult physiology.

To circumvent these limitations, the discovery and application of the bacteriophage P1 *Cre/LoxP* system recombination and the *Saccharomyces cerevisiae* recombinase *FLP/FRT* system have enabled the development of conditional knockouts. This strategy of gene targeting allows inactivation of an endogenous gene in the mouse to be controlled in both a temporal and spatial manner. These two systems rely on the function of integrase site specific recombinases, *Cre* and *FLP* recombinase, which specifically recognise the presence of 34 bp recognition sites, *loxP* and *FRT* respectively.

The *FLP/FRT* system is derived from yeast and relies upon the action of 43 kDa FLP recombinase that recognises the 34 bp *FRT* sites. This system has been successfully used to remove *FRT*-flanked selection cassettes in ES cells, however in comparison to the *Cre/LoxP* system, the *FLP/FRT* has been reported to work less efficiently in ES cells and transgenic mice (Fiering *et al.*, 1995; Buchholz *et al.*, 1996).

3.1.3 *Cre/LoxP* System

Cre is the 38 kda product of the *cre* (cyclisation recombination) gene of bacteriophage P1 and is a site-specific DNA recombinase. *Cre* recognises a 34 bp site on the P1 genome called *LoxP* and efficiently catalyses reciprocal conservative DNA recombination between pairs of *LoxP* sites. *LoxP* sites consist of two 13 base pair inverted repeats (CRE recognition sites) separated by an 8 base pair spacer (Figure 3.3 (a)). If two *loxP* sites are orientated in the same direction, the intervening DNA will be excised, with one *loxP* site remaining (Figure 3.3 (b)). Conversely if two *loxP* sites are orientated to face each other, the intervening DNA will be inverted (Figure 3.3 (c)).

The first report demonstrating the use of *Cre* recombinase to mediate site specific recombination in transgenic mice was made by Lakso *et al* (Lakso *et al.*, 1992). In this study the *Cre/LoxP* system was used to activate a dormant transgene in mice. It had been previously demonstrated that directed expression of the simian virus SV40 large tumour antigen (SV40 large T antigen), in the murine lens, induced the development of malignant tumours (Mahon *et al.*, 1987). A construct was designed in which a STOP sequence flanked by *loxP* sites was inserted between the lens specific promoter (alpha A-crystallin) and the SV40 large tumour antigen gene sequence. This STOP sequence prevented the expression of the SV40 large T antigen. There was no detection of the expression of the SV40 large T antigen in transgenic mice carrying this construct and all exhibited normal development of the lens. These transgenic mice were then crossed onto *Cre* expressing line of transgenic mice which *Cre* expression was under the control of either the murine alpha A-crystallin promoter or the human cytomegalovirus promoter (CMV). In all double transgenic offspring, mice developed tumours in the lens. Further analysis confirmed that tumour formation was a result of SV40 large T antigen expression activated by the *Cre*-mediated deletion of the stop sequence (Lakso *et al.*, 1992).

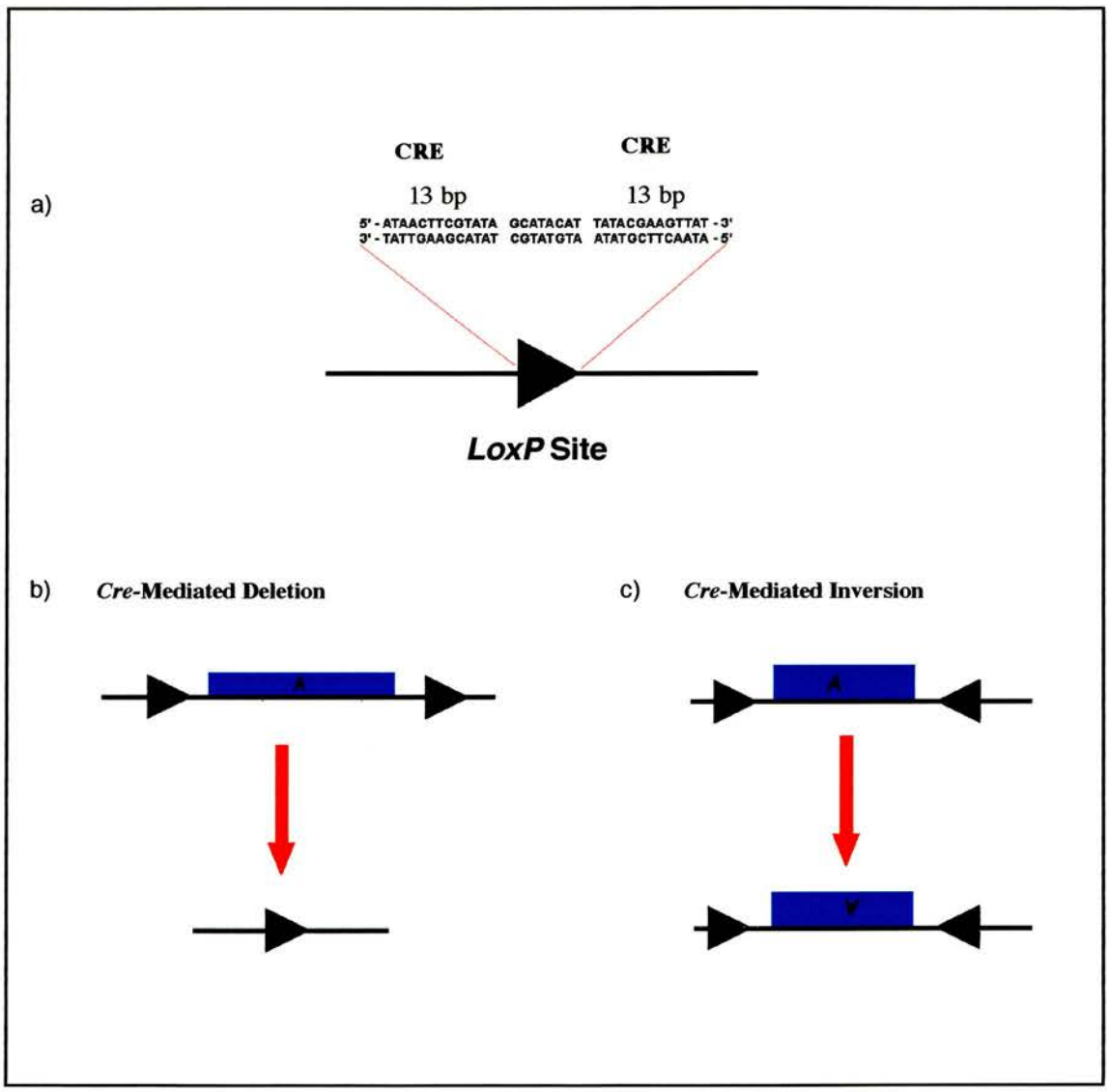


Figure 3.3 The *Cre/Lox*P System

a) Structure of the *LoxP* site. The *LoxP* site (34 bp) consists of two 13 bp inverted repeats and an asymmetric 8 bp spacer region.

b) *Cre* -Mediated Deletion. *LoxP* sites placed in the same orientation, following *Cre* recombination, results in excersion of the intervening DNA, with one *loxP* site remaining.

c) *Cre* -Mediated Inversion. *LoxP* sites placed in the opposite orientation to each other, following *Cre* recombination, results in inversion of the intervening DNA.

The first reported example of conditional ablation of an endogenous gene through *Cre/LoxP* targeting in ES cells was the conditional ablation of DNA polymerase β gene (polβ) in T cells (Gu *et al.*, 1994). Conventional knockout of the DNA pol β gene results in embryonic lethality, therefore *loxP* sites were inserted either side of the first exon of the DNA pol*β* gene by homologous targeting in ES cells (Gu *et al.*, 1994; Sugo *et al.*, 2000). Crossing the DNA polβ^{flox} mice onto a thymocyte-specifc *Cre* expressing transgenic mouse line, resulted in specific ablation of DNA polβ in mature T cells. To date there have been numerous reports of genes in which a conditional knockout approach through homologous recombination in ES cells, using the *Cre/LoxP* system, has answered many important biological and development questions.

3.1.4 Examples of Conditional Knockouts using *Cre/LoxP*

3.1.4.1 Nkx3.1 Induces Prostatic Intraepithelial Neoplasia

The homeodomain-containing transcription factor Nkx3.1, is a putative tumour suppressor that is expressed in a largely prostrate-specific and androgen-regulated manner (Bieberich *et al.*, 1996; Bhatia-Gaur *et al.*, 1999). Although no mutations in the *Nkx3.1* gene have been found in a survey of prostate tumour specimens, loss of Nkx3.1 protein expression has been observed in ~40% of human prostate tumours and in ~20% of prostatic intraepithelial neoplasia (PIN) lesions and correlates with tumour progression (Voeller *et al.*, 1997; Bowen *et al.*, 2000). The *Nkx3.1* gene also maps within the minimal deleted region of 8p21 that is lost in prostate tumours making it a strong candidate for a prostrate tumour suppressor gene (Voeller *et al.*, 1997). As Nkx 3.1 plays a key role in the development and differentiation of the prostatic epithelium, it provides an important link between development and carcinogensis in the prostate gland.

In the mouse, conventional knockout of the *Nkx3.1* gene by insertion of a neomycin cassette, leads to developmental defects of the prostate gland, including defects in ductal branching morphogenesis, prostatic secretions, and

epithelial hyperplasia and dysplasia (Bhatia-Gaur *et al.*, 1999). The prostatic epithelial hyperplasia and dysplasia that develops in Nkx3.1 null mice supports a role for Nkx3.1 in growth suppression in the prostatic epithelium. However this phenotype prevents any further analysis of the precise role of Nkx3.1 in prostatic carcinogenesis.

Using *Cre/LoxP* mediated recombination in adult prostate, conditional ablation of one or both alleles of Nkx3.1 leads to the development of preinvasive lesions that resemble human features of PIN (Abdulkadir *et al.*, 2002). Therefore using this system, the authors were able to circumvent the developmental defects resulting from the absence of Nkx3.1 and demonstrate a role for Nkx3.1 as a prostate tumour suppressor and also provide evidence to support its function in tumour initiation.

3.1.4.2 Rapid Colorectal Adenoma Formation in Conditional APC Mice

Mutations in the *APC* gene have been demonstrated to be responsible for Familial Adenomatous Polyposis coli (FAP), a disease characterised by the development of multiple colorectal adenomas (Nishisho *et al.*, 1991; Joslyn *et al.*, 1991). Analysis of colorectal adenomas from FAP patients has shown that although they carry germline mutations in the *APC* gene, their tumours show inactivation of the wild-type allele *(*Ichii *et al., 1993).* Inactivation of both copies of the APC gene also occurs frequently in sporadic colorectal adenomas (Miyoshi *et* al., 1992; Smith et al., 1993). However although this data strongly suggests that *APC* is acting as a tumour suppressor, the mechanisms of transformation of colorectal epithelial cells to adenomas following the inactivation of APC remain unclear.

Several mouse models carrying mutations in the *APC* gene have been established as experimental models for FAP (Su *et al.*, 1992; Fodde *et al.*, 1994). However complete deficiency in APC results in embryonic lethality (Moser *et al.*, 1995. Conditional gene targeting using the *Cre/LoxP* system

allowed the development of a mouse model for APC ablation specifically to be carried out in colorectal epithelial cells (Shibata *et al.*, 1997). *LoxP* sites were targeted into the introns surrounding exon 14 of the *APC* gene. Mice homozygous for *loxP* modifications were phenotypically normal. However, upon infection of the colorectal region with an adenovirus expressing *Cre* recombinase, the mice developed colorectal adenomas within 4 weeks. Analysis of the adenomas demonstrated an ablation of exon 14 of the *APC* gene. This system therefore provides a mouse model for the investigation of the steps in adenoma formation, which develop from the specific inactivation of APC.

3.1.5 Current Mouse Model of WT1 Loss-of-Function

To determine the role of WT1 in development, a replacement type targeting vector was used to replace the first exon of *WT1* and 0.5 kb upstream sequence of *WT1* with a neomycin resistance gene (Kreidberg *et al.*, 1993). Heterozygote mice appeared phenotypically normal up to 9 months of age. Homozygous WT1 null mice however died between E13 and E15 due to developmental defects of the heart (Kreidberg *et al.*, 1993). The WT1 null mice also completely lack kidneys and gonads. The arrested development of the kidney and gonad at a very early stage prevents any analysis of the role of WT1 in later stages of nephrogenesis and gonadogenesis. Also the embryonal lethality of the WT1 null mice, makes the analysis of the role of WT1 in the later stages of development and adult physiology impossible.

Therefore an alternative approach to investigate the role of WT1 in embryogenesis, urogenital development and adult physiology is to conditionally inactive WT1. As shown by the examples above, the *Cre/LoxP* system is a powerful approach that can be applied to ablate WT1 in a spatial and temporal manner. This system will permit questions to be directly asked about the role of WT1 in development and disease.

3.2 Strategy for Conditional Knockout of WT1

The strategy chosen to generate a conditional allele of *WT1* is to flank exon 1 with *loxP* sites such that *Cre* recombinase will delete exon 1 to produce a null allele. This will be similar to the original mutation produced by Kreidberg *et al*, who replaced a similar genomic interval with a neomycin gene to produce a *WT1* null allele (Kreidberg *et al.,* 1993*)* (Figure 3.4). In the absence of *Cre*, targeted clones will be identical to the wild-type allele apart from the presence of the 5' and 3' *loxP* sites (34 bp each). Upon exposure to *Cre* recombinase, a null allele will be produced due to the excision of DNA sequence encoding for exon 1. This genomic deletion removes the transcriptional and translational start sites that lies between the two *loxP* sites producing a null *WT1* allele.

3.2.1 Targeting Vector and Targeting of Embryonic Stem Cells

The design of the targeting construct and initial ES cell homologous targeting was carried out by Dr. Lustig-Yariv. Briefly, the construct was obtained from screening a 129/Ola BAC genomic library. A 6 kb *BglII* fragment was isolated encompassing 2.5 kb upstream of exon 1 and 2 kb of intron 1 and inserted into *pBluescript*. Synthetic oligonucleotides were inserted at the *AatII* site, 0.5 kb upstream of exon 1, which coded for a *loxP* site. An expression cassette containing the Herpes Simplex thymidine kinase (*HSVtk*) and neomycin (*neo*) genes, each under the control of the phosphoglycerate kinase promoter (*Pgk-HSVtk-Pgk-Neo*), flanked by *loxP* sites, was inserted 3' of exon 1 into the *EcoRV* site 1 kb downstream of exon 1, to produce the full targeting construct, *pWT1LoxP24*. The three *loxP* sites in *pWT1LoxP24* were present in the same orientation to enable *Cre*-mediated deletion of the intervening DNA (Figure 3.4).

The construct was designed to allow a 2-step strategy to be employed. The first step is to target the *WT1* endogenous locus with the *pWT1LoxP24* construct by homologous recombination in ES cells, introducing the *loxP* modifications. This

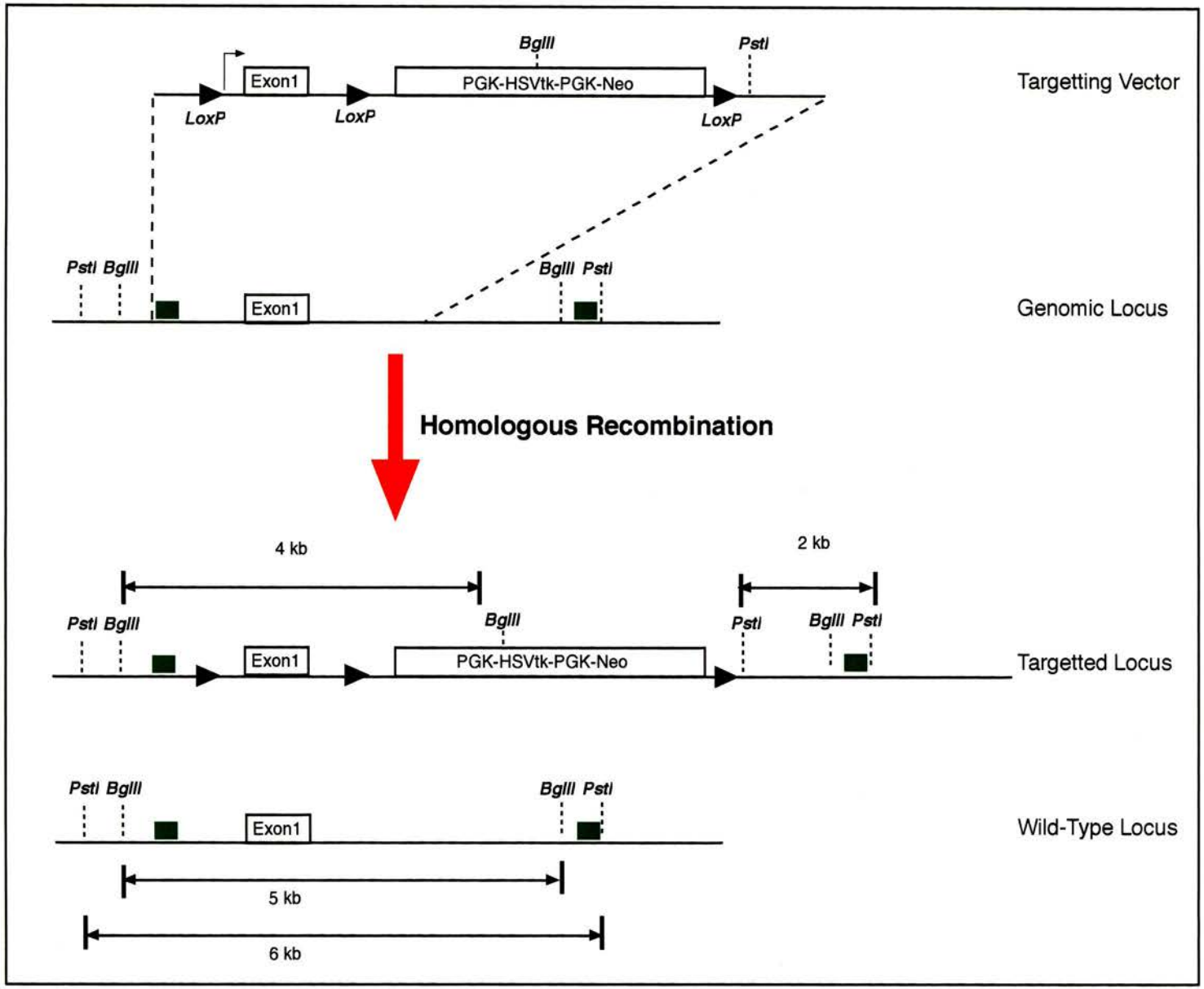


Figure 3.4 Schematic of the Targeting Construct (*pWT1LoxP24*) Used in the Generation of a Conditional Allele of *WT1*

A 6 kb *bglII* fragment spanning exon 1 of *WT1* was modified to incoporate a floxed PGK-TK-PGK-Neo selection cassette. A *loxP* site was placed upstream of the transcriptional start site for WT1 to produce the 11 kb targeting vector, *pWT1LoxP24*. Marked on the figure are the restriction sites and probes used in the Southern blot hybridisations (green boxes).

step relies on positive selection, in this case provided by the presence of a neomycin gene and selection in G418. The next step is to remove this selectable cassette. Following this step the ES cells will revert back to a targeted allele that will be identical to the wild-type allele, apart from the presence of the 5' and 3' *loxP* sites. To achieve this the neomycin cassette was included in the *pWT1LoxP24* targeting vector as part of a *loxP* flanked cassette containing the *HSVtk* gene. This *Pgk-HSVtk-Pgk-Neo* selection cassette allows for a positive-negative selection procedure. Homologously targeted clones are transiently transfected with a *Cre* expression vector and colonies resistant to gancyclovir (TK^{-ve}) are isolated, in which the double selectable cassette has been excised.

The linearised targeting vector *pWT1LoxP24* was electroporated into in E14 (129/Ola) ES cells. Cells were selected with G418 for 10 days. 290 clones were picked which survived G418 selection and from these 190 were screened via Southern Blot, using a *PstI* genomic digest and 3' external probe, i.e. outside the region of homology of the targeting vector. From the 190 screened, 5 clones were identified as being homologously targeted at the WT1 locus (12.5, 13.5, 14.17, 17.5, 20.4) using the 3' external probe.

These 5 clones were subjected to Southern blot analysis to confirm the earlier findings made by Dr. Lustig-Yariv. A Southern blot using a *BglII* restriction digest of genomic DNA and a probe located upstream of the 5' *loxP* site and exon 1 produced the 5 kb band and 4 kb band fragments expected for homologous recombination (Figure 3.6 (b)). A Southern blot was then performed using a probe external to the 3' end of the targeting vector on *PstI* digested genomic DNA. Positive clones were detected with a 5 kb wild-type and 2 kb targeted fragment arising from the insertion of a novel *PstI* restriction site within the targeting vector (Data not shown). The use of the 2 probes verifies that there is one site of insertion for targeting vector and that this is at the *WT1* endogenous locus.

3.2.2 Generation of WT1 Floxed ES Clones

The next step is to take the homologously targeted ES clones from the fist round of targeting and to remove the selectable marker. This will generate ES clones in which only *loxP* sites either side of exon 1 would remain. These ES clones would then be used to inject blastocysts and generate the conditional WT1 knockout line of mice.

Transient transfection of a homologously targeted clone with *pMC-Cre* plasmid results in the partial recombination between the 3 *loxP* sites. From the transfection three possible outcomes are possible: Type 1 recombination (recombination between *loxP* sites flanking the selection cassette) produces clones in which the selection cassette is excised, leaving the *WT1* exon 1 flanked by *loxP* sites. Type II recombination (recombination between the two outer *loxP* sites) removes the full genomic interval between the 3 *loxP* sites which includes exon 1 and the selection cassette, leaving effectively a null allele. Type III recombination (recombination between the *loxP* sites surrounding exon 1) generates clones where exon 1 is excised, leaving the selection cassette at the locus (Figure 3.5). Using negative selection with GANC following the transient transfection with *Cre*, clones that undergo type III recombination (*HSVtv*$^{+ve}$) are non-viable as they will not survive the GANC selection due to the presence of the *HSVtk* gene. Similarly, ES cells that have not been transfected with the *pMC-Cre* plasmid, are also non-viable as they will also retain the *HSVtk* gene. Therefore following *Cre* treatment and efficient gancyclovir selection, cells selected and screened will possess either an exon 1 allele, flanked by *loxP* sites, or a complete deletion of the genomic sequence spanning the exon 1 locus.

Homologously targeted ES clone, 14.17, was transiently transfected with supercoiled *pMC-Cre* and 30 gancyclovir resistant clones were analysed by Southern blot using a *BglII* digest of genomic DNA and a probe located

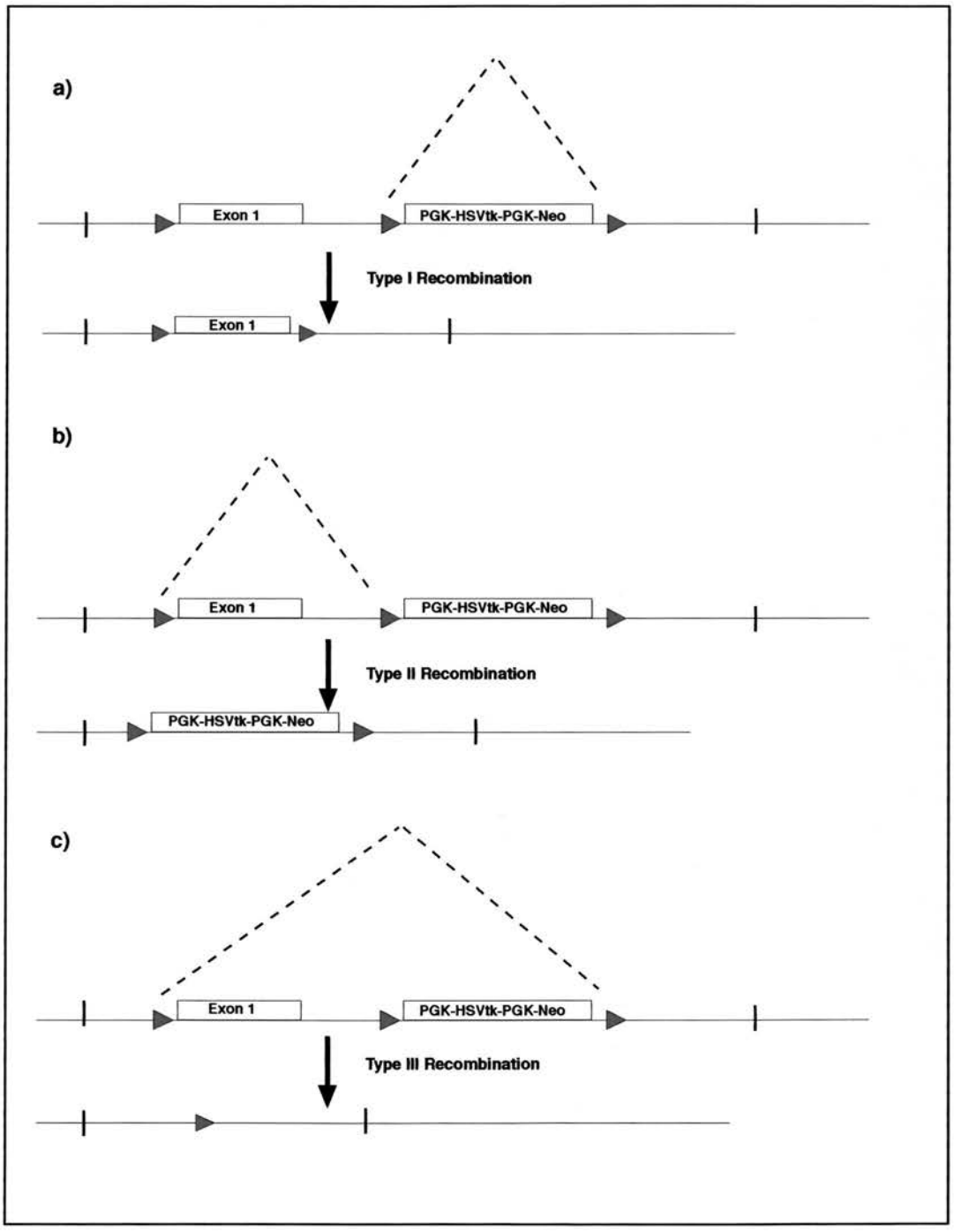


Figure 3.5 Effects of *Cre*-Mediated Recombination at the Targeted *WT1* Locus

a) Type I Recombination: Recombination between the *LoxP* sites flanking the selection cassette

b) TypeII Recombination: Recombination between the *LoxP* sites flanking Exon 1 of *WT1*

c) Type III Recombination: Recombination between the outer *LoxP* sites

upstream of the 5' *loxP* site and exon 1. From the transfection, both type 1 and type II recombination events were detected (Figure 3.6). There was no detection of clones that had undergone type III recombination. Southern blot analysis would have detected bands of 5 kb and 3 kb for this event. Similarly no clones were detected that had not undergone *Cre* mediated recombination. Therefore the identification of clones harbouring only type I and type II recombination demonstrates that the *Cre-loxP* system and the drug selection is working efficiently.

3.2.3 Generation of Chimeras and Germline Testing

Two independent ES clones that contained exon 1 flanked by *loxP* sites, *WT1-LoxP*.3 and *WT1-LoxP*.26, were injected into C57/BL6 blastocysts to generate chimeric mice. The blastocyst injections were carried out by Dr. Sheila Webb. From the injections, 5 chimeras were obtained, in which 4/5 were male, indicating a good contribution to blastocyst development by the male ES cell clones. From the coat colour it was also apparent that the level of chimerism was high, ranging from 60-80%.

The 4 male chimeric mice were tested for germline transmission of the targeted allele. Male chimeric mice were mated with C57/BL6 female mice. From these matings, transmission of ES (129/Ola) cells can be identified by the presence of agouti offspring. Table 3.1 shows the breeding of the chimeras onto a C57/BL6 background.

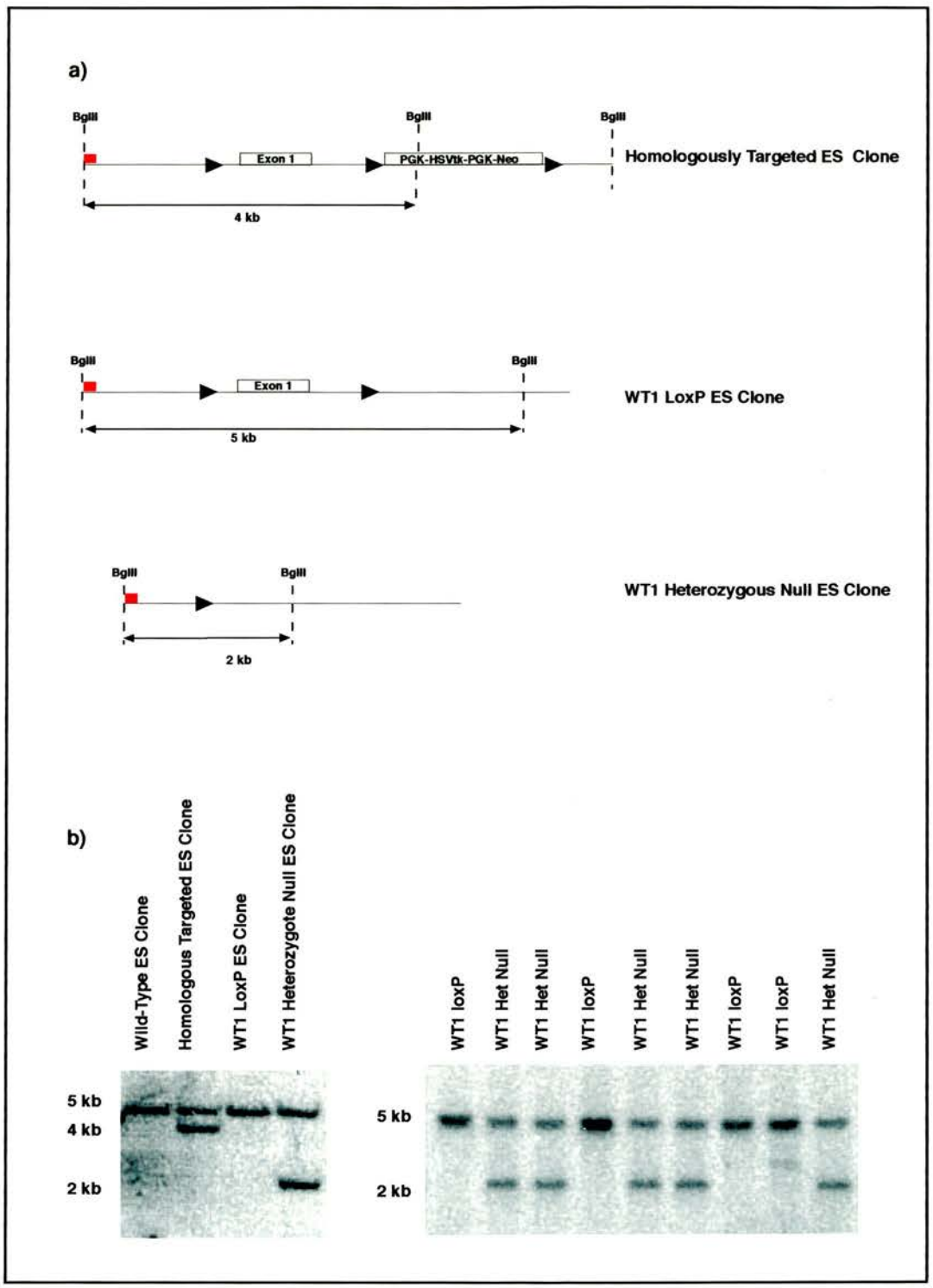


Figure 3.6 Southern Blot Analysis following *Cre*-Mediated Recombination of ES Cell Clone 14.17

a) Diagram showing the Southern blot used to analyse ES clones following *Cre*-mediated recombination

b) 10 μg of genomic DNA was digested with *BglII*. Digested DNA was Southern blotted and then probed with he 5' probe (red box). ES clones carrying the full targeting vector, generate 5 kb and 4 kb bands, floxed ES clones generate a 5 kb band and heterozygous WT1 null ES cells generate 5 kb and 2 kb fragments.

Chimera	Number Live Births	Number Agouti Offspring
Chimera 16	**12**	**1**
Chimera 17	**46**	**4**
Chimera 18	**127**	**16**
Chimera 19	**59**	**2**
Chimera 20	**11**	**0**

Table 3.1 Breeding of *LoxP* Chimeric Mice

From the breeding of chimeras, 23 agouti offspring were obtained from 255 live births. To follow transmission of the targeted allele, a PCR screen was used to detect the presence of the 5' *loxP* site. Primers were designed to span the region in which the 5' *loxP* site had been inserted. The wild-type allele generates a PCR product of 200 bp whilst the *WT1-LoxP* targeted allele will generate a 234 bp PCR product, indicating the presence of the *loxP* site. Analysis of the agouti offspring indicated that none were carrying the *WT1-LoxP* targeted allele, as all displayed the wild-type 200 bp product (Figure 3.7 (a)). This was surprising, as one would expect 50% of the agouti offspring to be transgenic.

Due to the lack of transmission, skin biopsies were taken from chimeras 18 to confirm that the chimeras were generated from correctly targeted ES clones. A PCR screen using the primers spanning the 5' *loxP* site showed that the chimera was generated from correctly targeted ES cells, as 2 of the biopsy samples were positive for presence of the 5' *loxP* site (Figure 3.7 (b)).

Although the litter sizes from the C57/BL6 test matings were normal, the presence of agouti offspring was a rare occurrence, with only 1 or 2 agouti mice per litter of 6-8. Therefore could the absence of ES cell derived mice carrying the *WT1* targeted allele indicate that the modifications to the *WT1* locus might be causing embryonic lethality? In planning where to place the *loxP* sites in the

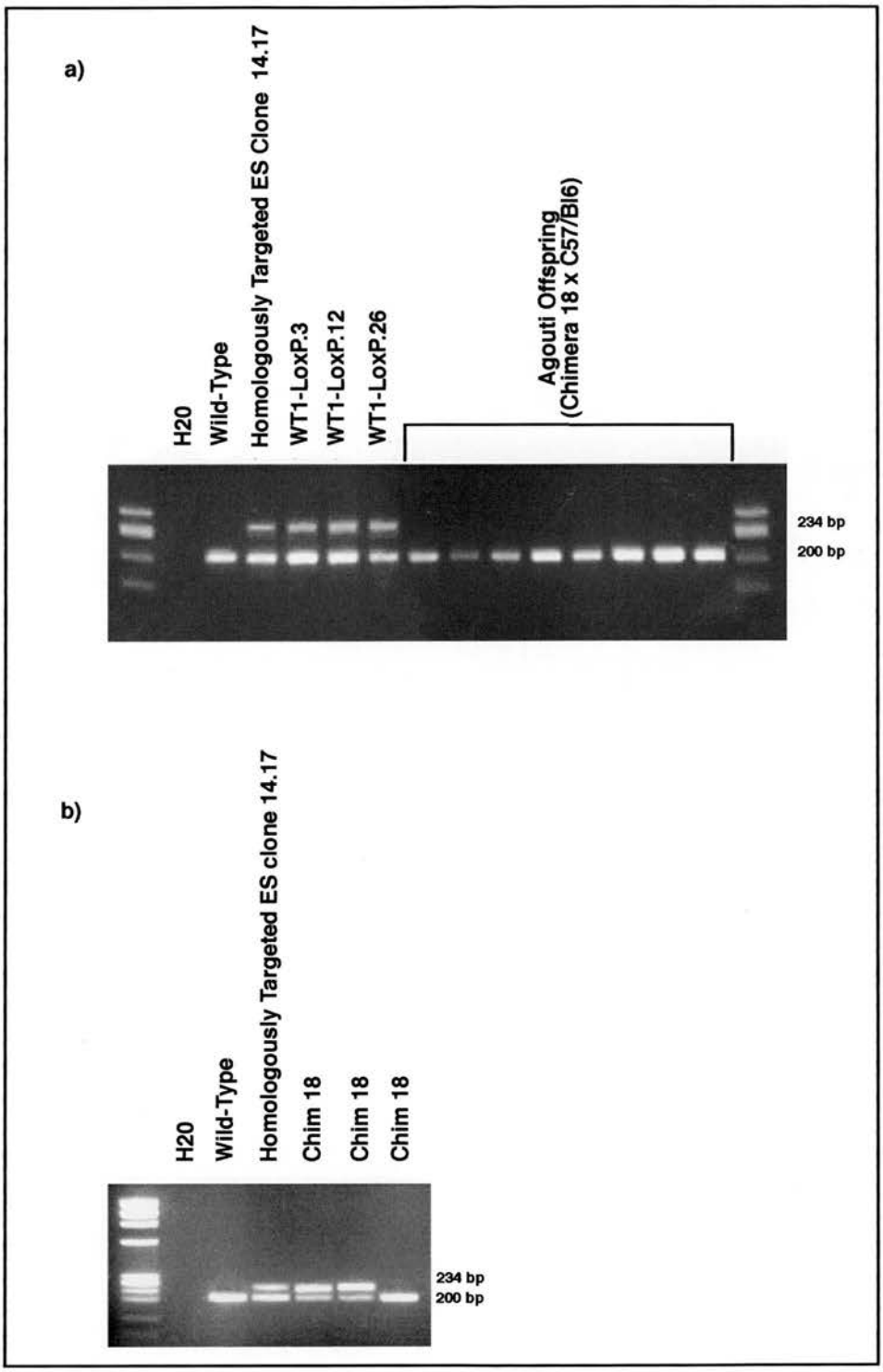


Figure 3.7 PCR analysis of the 5' *LoxP* Site

a) PCR analysis for the 5' *LoxP* site of agouti offspring from the breeding of chimera 18 with female C57/Bl6, indicates that none carry the targeted *WT1* allele.

b) PCR analysis for the 5' *LoxP* site from skin biopsies of chimera 18, demonstrates that the chimeras are generated from correctly targeted ES cells, which carry a *loxP* site at the *WT1* locus.

pWT1LoxP24 targeting vector, care was taken to place the *loxP* sites in genomic regions that were not highly conserved between mouse and human (Figure 3.8). Added to this, the *loxP* sites were orientated in the genome to avoid adding an extra translational ATG start site into the *WT1* locus, as this sequence was present within the *loxP* sites. The presence of the 3' *loxP* site can be excluded from having a negative effect on germline transmission of the targeted allele, as this *loxP* site was present in ES cells generated for the ablation of the WT1 CTG isoforms (Miles *et al.*, 2003). In the targeting of ES cells to ablate the CTG isoform, *Cre/LoxP* was used to remove the selectable cassette following homologous recombination. Once the selectable cassette was removed following transient expression of *Cre*, a single *loxP* site remained in the first intron of *WT1*. Chimeras generated from this targeting event produced germline transmission of the CTG targeted allele (Miles *et al.*, 2003). Therefore could the problem lie in the position of the 5' *loxP* site? This *loxP* site lies 0.5 kb from the start site in the 5' UTR region. Could the 5' *loxP* site be adversely affecting transcription of the *WT1* targeted allele? Even if there were an affect of placing the *loxP* site in the 5' position, this would have to produce a dominant effect at the targeted allele producing a hypermorphic *WT1* allele or induce ectopic expression of WT1 at the targeted *WT1* locus to cause embryonic lethality. This is because the WT1 heterozygote null mutation does not result in embryonic lethality and WT1 heterozygote null mice are generated at the expected Mendelian ratio (Kreidberg *et al.*, 1993). Therefore it was decided to carry out detailed analysis of ES clones *WT1-LoxP.3* and *WT1-LoxP.26* that were used to generate the chimeras.

As already demonstrated in figure 3.7, the two clones were correctly targeted for the 5' *loxP* site. However using a PCR for the 3' *loxP* site, with primers that should generate a PCR product of 200 bp for wild-type and 234 bp product for 3' *loxP* targeted alleles, only a wild-type product of 200 bp could be detected in the 2 clones (Figure 3.9 (a)). This was in contrast to a DNA sample from the CTG knockout mice, where the 3' *loxP* site lies in the same genomic position in

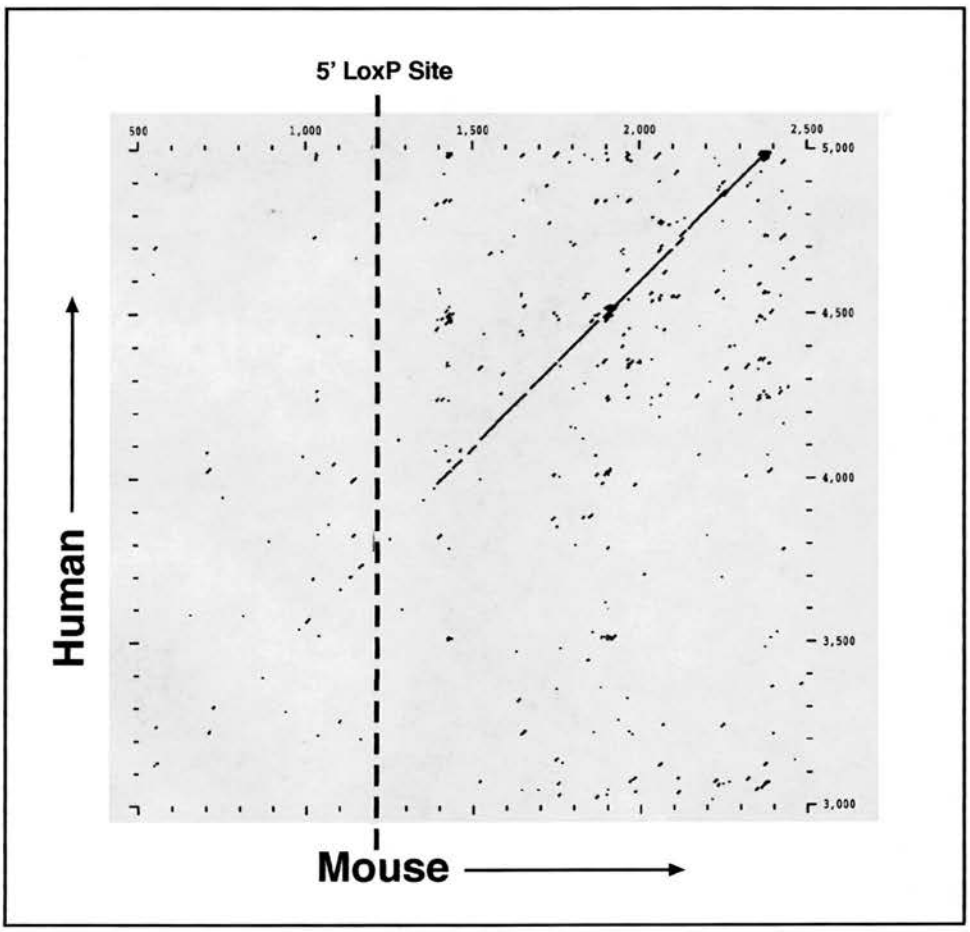


Figure 3.8 DOTPLOT comparison of Human and Mouse *WT1* Genomic Sequence Upstream of the ATG

DOTPLOT comparison of the human and mouse *WT1* genomic sequence surrounding the region in which the 5' LoxP site is positioned. This indicates that the 5' LoxP site is positioned in a genomic region that is not conserved between human and mouse.

intron 1 of the *WT1* locus. PCR on DNA from CTG knockout heterozygote and homozygote mice clearly shows the present of the 3' *loxP* site and the wild-type allele in the heterozygote mice, whilst in the homozygote mice this reverts to a single product of 234 bp indicating both alleles contain the 3' *loxP* site.

A Southern Blot was then performed using a probe external to the 3' end of the targeting vector on *PstI* digested genomic DNA. As shown previously, the first round of targeting produces homologously targeted clones with an insertion of a novel *PstI* restriction site within the targeting vector. This produces 6 kb and 2 kb fragments on a *PstI* digested genomic DNA Southern blot. In the *WT1-LoxP* clones the same 6 kb wild-type and 2 kb bands should still be present, as the novel *PstI* site lies outside of the region where *loxP* recombination occurs. When both *loxP* clones were analysed using this Southern blot, only the wild-type 6 kb band could be detected on the Southern Blot (Figure 3.9 (b)).

Therefore both the 3' *loxP* PCR and the Southern Blot indicated that the region surrounding the 3' *loxP* site has been disrupted during the *Cre* mediated recombination of the parental clone, 14.17. This was an unexpected finding as *Cre* mediated recombination has been not been reported to be an inaccurate process.

3.2.4 Generation of *WT1-LoxP* ES clones from 12.5 and 20.4

Due to the problems associated with *WT1-LoxP* clones derived from the *Cre* treatment of 14.17, another 2 homologously targeted clones, carrying the full targeting vector, were selected for *Cre* treatment (12.5 and 20.4).

From transfections of clones 12.5 and 20.4, 30 subclones in total were isolated following selection in gancyclovir for 8 days. Clones were firstly screened by Southern blot using a *BglII* restriction digest of genomic DNA and hybridisation with a 5' probe. As with the analysis of 14.17, no clones from the transient

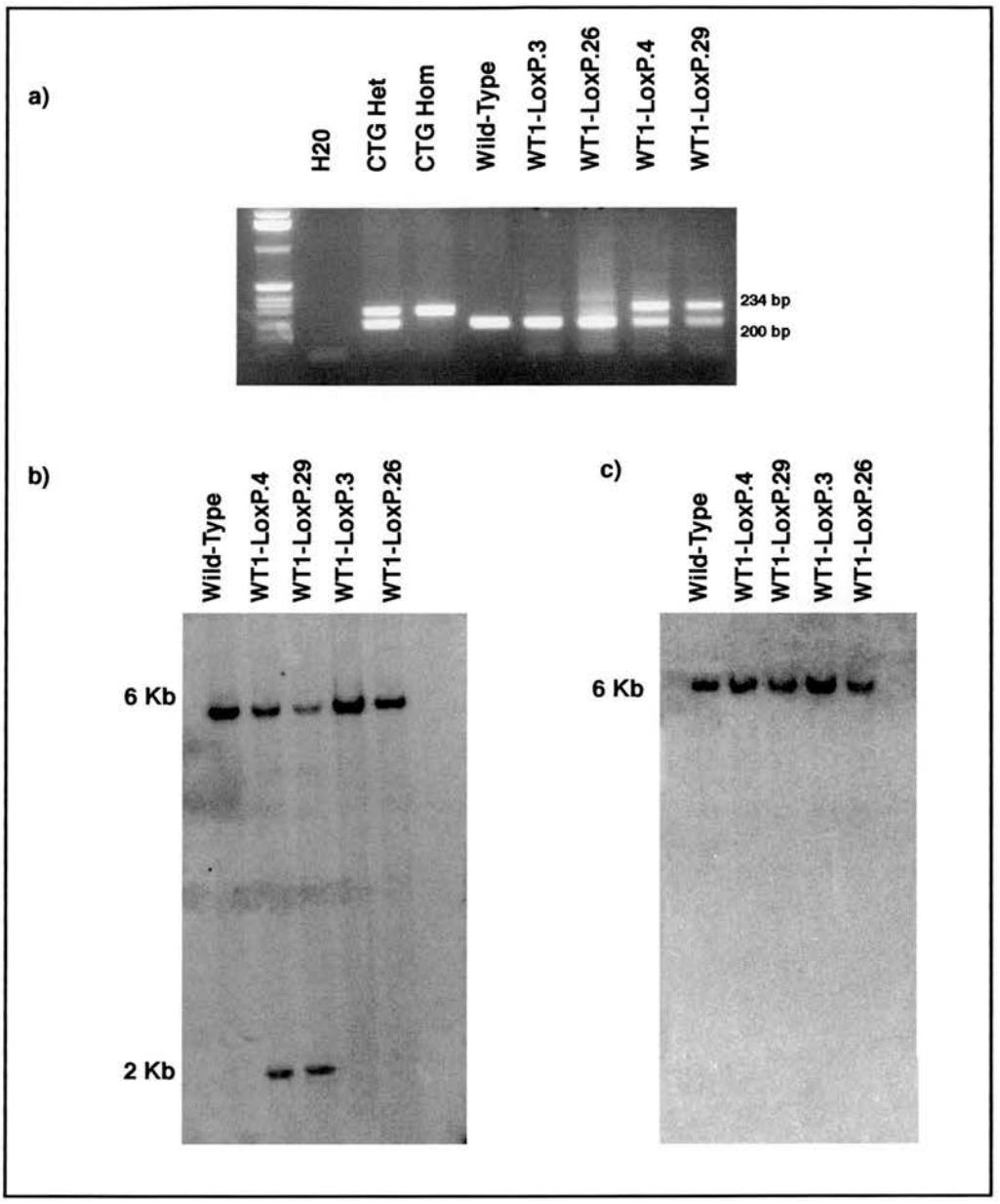


Figure 3.9 Analysis of the 3' *LoxP* Site by Southern blot and PCR Demonstrates Disruption of the Genomic Region following *Cre*-mediated Recombination

a) PCR analysis of the 3' *LoxP* site in floxed ES clones used to generate chimera 16-20 (from ES clones WT1-LoxP.3 and WT1-LoxP.26) demonsrates that following *Cre*-mediated recombination, the genomic region surrounding the *LoxP* site is disrupted. In contrast, the floxed ES clones used to generate chimeras 54 and 55 (from ES clones WT1-LoxP.4 and WT1-LoxP.29) following *Cre*-mediated recombination, do contain a 3' *LoxP* site, as detectable by PCR.

b) Southern blot analysis of *PstI* digested genomic DNA probed with a 3' probe, confirms that the unique *PstI* site present in the targeted *WT1* locus, is lost in the WT1-LoxP.3 and WT1-LoxP.26 ES clones, which were generatedfollowing *Cre*-mediated recombination of homologously targeted ES clone 14.17. In contrast, floxed ES clones (WT1-LoxP.4 and WT1-LoxP.29) generated from *Cre*-mediated recombination of homologously targeted ES clones 20.4 and 12.5, retain the unique *PstI* site.

c) Southern blot analysis of *BglII* digested genomic DNA probed with a 5' probe, confirms that all of the ES clones following *Cre*-mediated recombination, have lost the selectable marker cassette.

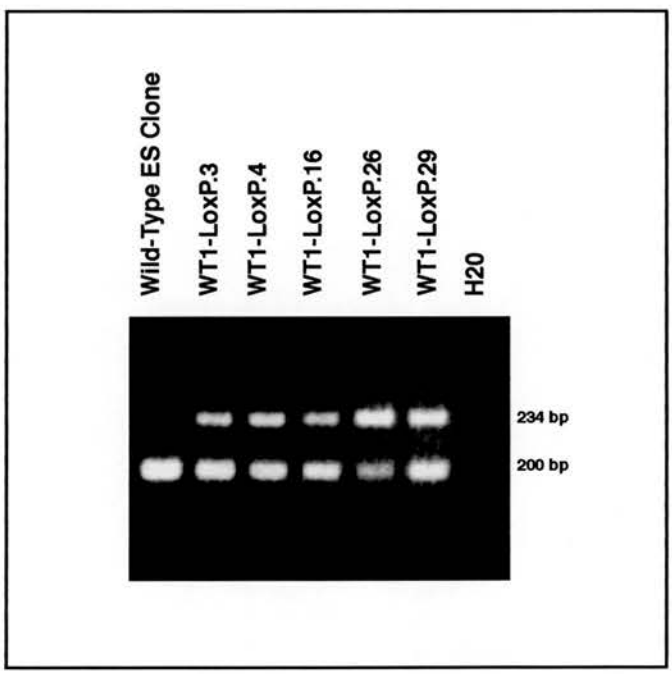


Figure 3.10 PCR analysis of the 5' *LoxP* site

PCR for 5' *LoxP* site in the floxed ES clones used to generate chimeras 16-20 (WT1-LoxP.3 and WT1-LoxP.26) and chimeras 54 and 55 (WT1-LoxP.4 and WT1-LoxP.29), indicates that all ES clones contain a *loxP* site upstream of the ATG start site.

transfection of 12.5 or 20.4 exhibited type III recombination. Similarly all clones had undergone *Cre* mediated recombination as none contained the complete targeting vector. Two clones were selected, in which type I recombination has occurred, *WT1-LoxP.4* and *WT1-LoxP.29* for further analysis.

Firstly these two clones were analysed using the PCR screen for the presence of the 5' and more importantly the 3' *loxP* site. PCR for the 5' *loxP* site showed that both clones carried the correct insertion of the *loxP* site into the *WT1* locus as shown by the presence of the wild-type 200 bp and the *loxP* targeted 234 bp product (Figure 3.10). PCR for the 3' *loxP* site also showed that both clones possessed the 3' *loxP* site as shown by the presence of the 200 bp product denoting the wild-type allele and also the 234 bp product for the *loxP* targeted allele (Figure 3.9).

Next the clones were analysed to ensure that the novel *PstI* site was still present. Both clones were screened by Southern blot using *PstI* digested genomic DNA and hybridised with the 3' external probe. Both clones showed the correct 6 kb and 2 kb fragments indicating that the *PstI* site was still present (Figure 3.9). Therefore from the PCR and Southern Blot analysis, the region surrounding the 3' *loxP* site is intact and had not been disrupted during the *Cre* mediated recombination step.

3.2.5 Generation of Chimeric Mice and Germline Transmission

Correctly targeted ES clones *WT1-LoxP.4* and *WT1-LoxP.29* were injected into blastocysts to produce chimeras. From the injections, 3 chimeras were obtained, 2 males and 1 female. Both male chimeras (chimera 54 and chimera 55) exhibited high levels of chimerism, estimated to be approximately 70%-80%. Chimera 54 (12.5) and chimera 55 (20.4) were mated with C57/BL6 females to test for germline transmission. Transmission of the ES cells through the germline was noted from both chimeras by the presence of agouti offspring.

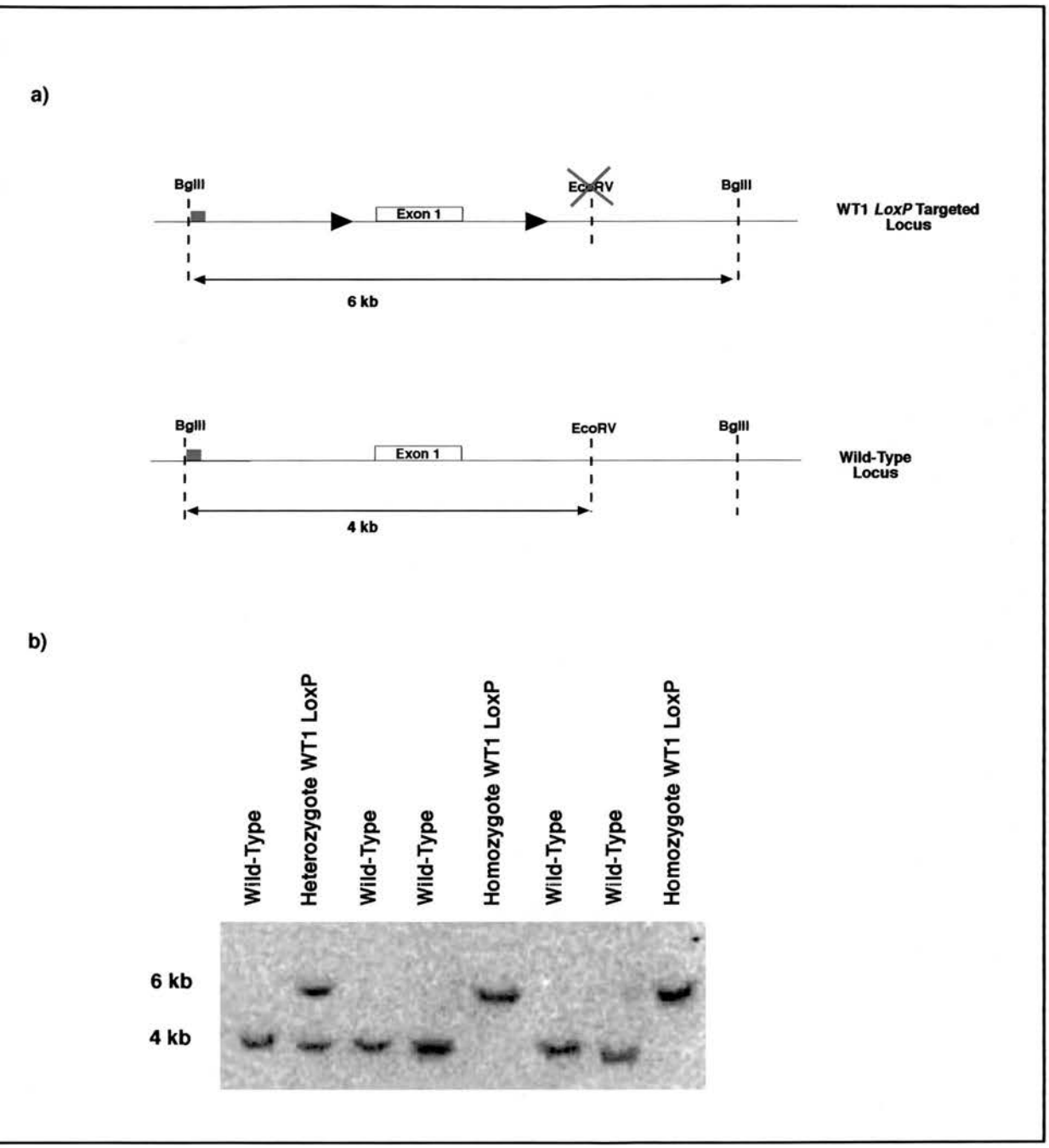


Figure 3.11 Germline Transmission of the Conditional *WT1* Targeted Allele

) Figure showing the Southern blot used to genotype the *WT1* conditional allele

) 10 μg of genomic DNA was digested with *BglII* and *EcoRV*. Digested DNA was probed by Southern blot with the 5' probe ed box) which distinguishes the *WT1* conditional targeted allele from the wild-type allele, on the basis of the absence of the ndogenous *EcoRV* site in the targeted locus. A wild-type *WT1* allele generates a 4 kb fragment, whilst a *WT1* conditional llele generates a 6 kb fragment.

Unlike the first set of chimeras where litters contained 1 or 2 agouti offspring in a litter of 6-8, the litters from both chimeras contained a high percentage of agouti offspring, with some litters containing 75% agouti pups.

The agouti offspring from both chimeras were genotyped by Southern blot on genomic DNA digested with *BglII* and *EcoRV* and hybridised with the 5' external probe (Figure 3.11 (a)). The Southern blot distinguishes the wild-type allele from the targeted allele, due to the insertion of the *HSVtk-Neo* selectable cassette into the endogenous locus during homologous recombination. Originally this selectable cassette was inserted in the targeting vector at an *EcoRV* site. This *EcoRV* site is destroyed during the cloning process to generate *pWT1LoxP24* and subsequently following homologous recombination in ES cells is destroyed in the *WT1* endogenous locus. Therefore a wild-type allele generates a 4 kb fragment, whilst correctly targeted allele generates a 6 kb fragment (Figure 3.11 (a)). Southern Blot analysis confirmed that both chimeras generated from the two independent clones had produced germline transmission of the *WT1* targeted allele (Figure 3.11 (b))

The next step was to cross the conditional WT1 heterozygote mice to homozygousity ensuring that the modifications to the *WT1* locus does not produce a phenotype in the offspring. Conditional WT1 heterozygotes, generated from the floxed ES *clone WT1-LoxP.29*, were intercrossed giving rise to normal Mendelian ratios of wild-type, heterozygote and homozygote offspring as determined by Southern Blot (Figure 3.11 (b)). Conditional WT1 homozygous mice were phenotypically normal up to 8 weeks of age.

3.3 Discussion

This chapter describes the targeting of the WT1 locus to establish a of a conditional knockout mouse model for WT1, using the *Cre/loxP* system. Although successful in obtaining a conditional WT1 mouse, difficulties were observed during the *Cre*-mediated manipulation *in vitro*. The first chimeras

generated from *loxP* flanked clones failed to produce germline transmission of the conditional allele. Subsequent analysis demonstrated that during the *in vitro* *Cre*-mediated recombination steps, there was a disruption to the genomic region which lies in the first intron of *WT1*. This was an unexpected finding, given that the process of *Cre* mediated recombination has not been reported to be an inaccurate process. Subsequent generation of chimeras from other two ES clones derived from an independent parental did not show any disruption of the genomic region surrounding intron one, and the conditionally target allele was successfully transmitted through the germline.

However caution must be taken in the interpretation of the data presented in this chapter with regards to the targeting of the WT1 allele. Although the original parental clones were characterised using two probes, the failure to use an external 5' probe, allows the possibility that the clones were not correctly targeted. Also an alternative explanation for the lack of transmission of the targeted allele may have been due to the possibility that the starting material used prior to the *Cre* treatment was a mixed clone, containing wild-type ES cells. Therefore upon gancyclovir selection a non-targeted population could have been enriched, and used in subsequent blastocyst injections. Future work will aim to confirm that a conditional allele has been established using the appropriate diagnostic Southern blots.

If successful, then this mouse model will provide an important resource for dissecting the *in vivo* role of WT1 in development and disease. The mouse model will enable the crucial questions to be asked regarding the function of WT1 during development. One on the most important issues is whether this mouse model can recapitulate the pathology of Wilms' tumour and provide a tool to investigate the development of the disease. Using suitable *Cre* lines, would conditional ablation of WT1 during this induction phase of nephrogenesis promote a predisposition to the onset of Wilms' tumours? In combination with inducible tissue specific *Cre* lines, the role of WT1 in the adult, including its expression in the podocytes and sertoli cells, can also be addressed. Therefore the creation of a conditional knockout for WT1 in

combination with suitable *Cre* expressing lines will begin to tackle some of the questions surrounding the role, and function of WT1, in development and disease.

Chapter 4 A Role for WT1 in Neuronal Development

Chapter 4 A Role for WT1 in Neuronal Development

4.1 Introduction

The restricted and dynamic spatio-temporal *in vivo* expression pattern of WT1, and also the limited availability of cell lines that express WT1 at detectable levels, has hampered investigations into the function and regulation of WT1. The majority of studies have been carried out on cell lines that are engineered to ectopically express single WT1 isoforms or transformed tumour cell lines. Although they have provided insights into the function of WT1, these studies may not represent fully the *in vivo* situation, which may explain the often contradictory conclusions drawn from such studies (For review see Reddy and Licht, 1996; Little *et al.*, 1999; Lee and Haber, 2001).

The establishment of murine embryonic stem (ES) cell lines has led the way to many new experimental approaches in the field of mammalian development biology. ES cells are pluripotent cells derived from the inner cell mass of developing blastocysts (Evans and Kaufman, 1981). ES cells are derived without the intervention of any immortalising agent, do not undergo either crisis or senescence, and retain a diploid karotype. They can proliferate without apparent limit and can readily be propagated clonally. They are maintained undifferentiated *in vitro* in the presence of Leukemia Inhibitory Factor (LIF) (Williams *et al.*, 1988; Smith *et al.*, 1988).

ES cells retain the potential for multi-lineage differentiation and produce a range of well-differentiated progeny. The establishment of various *in vitro* differentiation protocols has permitted the differentiation of ES cells along neuronal, cardiogenic, myogenic, hematopoietic, epithelial, adipogenic and chrondogenic lineages (For review see Keller, 1995; Wobus, 2001). An important finding during the differentiation of ES cells is that tissue specific genes, proteins, ion channels and receptors are differentially expressed in a

pattern closely resembling the pattern observed during mouse embryogenesis. This has been exemplified by the commitment of ES cells to the haematopoietic lineages. The haematopoietic system of the mouse is established early during embryogenesis from the extraembryonic mesoderm of the yolk sac. Differentiating ES cells as embryoid bodies (EBs), it has been demonstrated that the initial stages of haematopoietic development within EBs occurs in a ordered pattern similar to that within the embryo (Keller *et al.*, 1993). Therefore the differentiation of ES cells *in vitro* provides a powerful model system for addressing questions related to lineage commitment. However it also offers several advantages over comparable approaches in the whole embryo.

Firstly, ES cells in culture provide access to populations of early precursors that are difficult if not impossible to access *in vivo*. An example of this is the study of oligodendrocyte development in the CNS. Oligodendrocytes are post-mitotic cells that myelinate axons in the vertebrate central nervous system (CNS). They develop from proliferating oligodendrocyte precursor cells (OPCs), which arise in restricted regions of the ventral ventricular zone of the developing brain and spinal cord. In the mouse they are first detected as a narrow band in the ventral neuroepithelium around E12.5-13. So far it has only been possible to purify OPCs from the rat optic nerve (Raff *et al.*, 1988; Barres *et al.*, 1994). However they cannot be purified in large enough quantities to carry out conventional biochemical assays. Added to this is the inability to isolate CNS progenitor cells that give rise to the OPCs. This prevents the analysis of the earliest steps of oligodendrocyte differentiation. Using a genetically modified ES cell line which permits the selection of neuroepithelial cells *in vitro*, and in combination with appropriate signal molecules that promote OPCs *in vivo*, Billon *et al* were able obtain large quantities of OPCs (Li *et al.*, 1998; Billon *et al.*, 2002). From the starting population of OPCs, they were able then to differentiate OPCs to obtain oligodendrocytes. Employing markers to follow the fate of OPCs to oligodendrocytes, they were able to show that they differentiate following a schedule that is similar to that observed *in vivo*.

Secondly, and highly relevant for this study of WT1, is the study of ES cells carrying targeted mutations of genes, which are normally embryonic lethal *in vivo*. The developmental potential of these ES cells can be determined in culture. An example of this is the study of the transcription factor, Serum Response Factor (SRF). SRF is a ubiquitously expressed MADS box protein, which mediates the rapid transcriptional response to extracellular stimuli, e.g. growth and differentiation signals (Norman *et al.*, 1988; Treisman *et al.*, 1994). It has been shown that SRF is essential for murine embryogenesis. SRF homozygous null ($SRF^{-/-}$) embryos die at around E6.0 at the onset of gastrulation, with a failure to form detectable mesoderm (Arsenian *et al.*, 1998). However what is unclear is whether $SRF^{-/-}$ embryonic cells are generally incapable of differentiating into mesodermal cells derivatives or that the embryos do not provide the required signals for mesoderm formation. In order to analyse this defect, $SRF^{-/-}$ ES cells were generated and the effect of loss of SRF was studied *in vitro* (Weinhold *et al.*, 2000). The authors were able to show that there was an impairment of mesodermal differentiation *in vitro*, and this could be modulated in a non-cell-autonomous fashion (Weinhold *et al.*, 2000).

4.1.1 Retinoic Acid Induced Differentiation of ES Cells

Retinoic acid (RA) is a potent inducer commonly used to trigger differentiation of ES cells (For review see Rohwedel *et al.*, 1999). *In vivo*, retinoic acid is derived from the two step metabolism of vitamin A to retinal and its subsequent oxidisation to retinoic acid. It has been demonstrated that retinoic acid is essential for normal embryonic development and the regulation of growth and differentiation of a diverse range of cell types. It functions in the development of several organs during embryogenesis, including spinal cord motor neurons (Sockanathan and Jessell, 1998), eye (Wagner *et al.*, 2000), heart (Niederreither *et al.*, 2001) and kidney (Batourina *et al.*, 2001). RA functions by acting as a ligand for nuclear receptors, which in turn transcriptionally regulates RA target

genes, by binding to retinoic acid response elements (RAREs) within their promoters.

The treatment of embryonal carcinoma (EC) cells and embryonic stem (ES) cells with *all-trans* retinoic acid (RA) has been shown to induce high levels of WT1 expression (Scharnhorst *et al.*, 1997). WT1 protein was first detected in P19 EC cells following 4 days of induction with RA, whilst WT1 mRNA was detectable after 3 days. The authors suggested that the slow kinetics of WT1 expression after treatment of EC cells with RA was a result of cell differentiation and the emergence of a specific WT1 expressing cell lineage within the system, rather than a direct transcriptional effect of RA.

Immunofluorescence of differentiating P19 EC cells, treated with RA as a monolayer for 6 days, showed that nuclear staining for WT1 was present in approximately 80% of the cells. In an attempt to identify if there was a specific lineage within the RA differentiated EC cells that were expressing WT1, double immunofluorescence was used with a small set of lineage specific markers. The study revealed subpopulations of WT1 positive RA differentiated EC cells co-expressed either endodermal, epithelial or astrocytes markers. Therefore this indicated that there was not a single predominant lineage present in the RA treated EC cells which expressed WT1. Overall this is an intriguing finding given the restricted expression pattern of WT1 *in vivo*. It also indicates an accumulation of populations of WT1 positive cells, which could be amenable to manipulation and detailed study, and could be representative of cell-types found during normal differentiation process in which WT1 is expressed.

The aim of this chapter was to extend the study of RA induction of WT1 described in EC cells to ES cells. The induction of WT1 in monolayer cultures of ES cells by RA has been reported previously, however no description of the lineages which express WT1 in this system have been described (Scharnhorst *et al.*, 1997; Wagner *et al.*, 2001).

4.2 Differentiation of ES cells with RA Leads to Expression of WT1

The first step was to ensure that RA does induce the expression of WT1 in monolayer ES cells cultures, and to also analyse further the kinetics of that expression. As the original study by Scharnhorst *et al* used *all-trans* retinoic acid at a concentration of 1μM, this concentration was used for all subsequent work presented in this chapter and thesis (Scharnhorst *et al.*, 1997).

Wild-type ES cells were seeded in normal LIF containing medium in triplicate. 24 hours later, the cells were washed three times in PBS and the medium replaced by a) – LIF media, b) –LIF media supplemented with RA, c) normal LIF containing media. The medium was changed every 24 hours and fresh RA supplemented medium was added to the cultures. Following 96 hours the cells were harvested and total RNA and nuclear protein prepared from each culture condition.

Northern blot analysis was carried out using a full-length murine *WT1* cDNA. To confirm equal loading of RNA samples, the blot was stripped and reprobed with a probe specific for β-Actin. As a positive control for WT1 expression, total RNA from the M15 cell line was also used. The M15 cell line is a polyoma transformed mouse mesonephric cell line that expresses endogenously high levels of WT1 (Larsson *et al.*, 1995). No signal for WT1 was seen in the RNA prepared from undifferentiated ES cells or in RNA prepared from ES cells in which the LIF had been withdrawn (Figure 4.1 (b)). In the RNA sample prepared from ES cells exposed to RA, a band of 3 kb corresponding to WT1 mRNA was present (Figure 4.1 (b)). The level of WT1 expression in the RA differentiated ES cells was comparable to that of the M15 cell line.

Next the expression of WT1 protein was analysed from each of the conditions. 40 μg of nuclear protein was separated on a 10% SDS PAGE gel and transferred

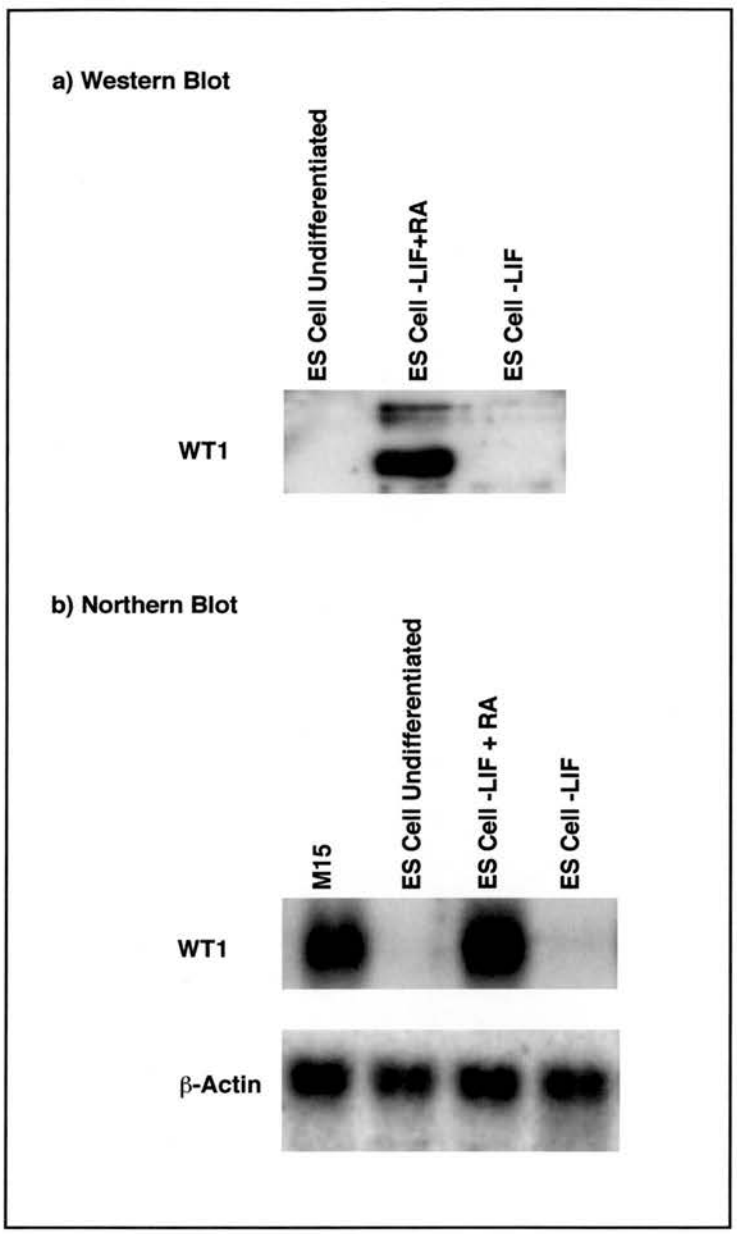


Figure 4.1 WT1 is Expressed in ES Monolayer Cultures Differentiated for 96 hours with *all-trans* Retinoic Acid (RA)

a) 40 μg of nuclear protein from undifferentiated ES cells, ES cells grown in media lacking LIF and ES cells grown in media lacking LIF and supplemented with 1μM of RA, was separated on 10% SDS PAGE gel and transfered onto nitrocellulose. The blot was probed for WT1 using the C-19 antibody.

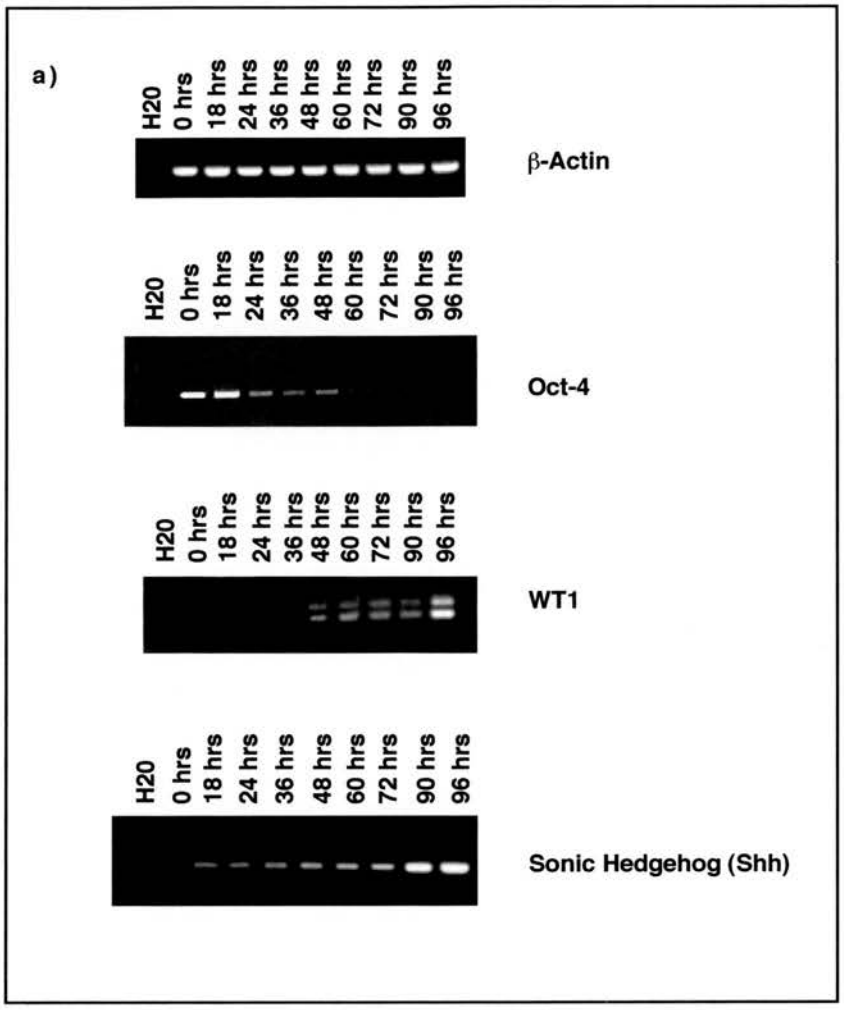
b) Total RNA from M15, undifferentiated ES cells, ES cells grown in media lacking LIF and ES cells grown in media lacking LIF and supplemented with 1μM of RA, were analysed by Northern blot. The blot was probed with a 32P labelled *WT1* cDNA probe. To control for loading, the blot was stripped and probed with a 32P labelled β-actin probe

onto nitrocellulose. An antibody for WT1, which is directed against the C-terminus of the protein (C-19), was used to probe the blot for the presence of WT1. No WT1 protein was detected in the undifferentiated ES cells or in the ES cells grown in the absence of LIF. However in the sample which was supplemented with RA, following 96 hours of differentiation, a characteristic doublet for WT1 at 52-54 kDa can be detected (Figure 4.1 (a)). These bands correspond to different WT1 isoforms and arise from alternative translation initiation sites and the presence/absence of the 17 amino alternatively spliced exon 5.

4.2.1 Kinetics of WT1 Expression

Having established that *all-trans* retinoic acid induces ES cells to express WT1, the kinetics of WT1 induction by RA were investigated. Wild-type ES cells were plated and induced to differentiate in the absence of LIF and in the presence of RA. Total RNA was collected at the following time points: 18 hours; 24 hours; 36 hours; 48 hours; 60 hours; 72 hours; 90 hours; 96 hours. 5μg of RNA was reverse transcribed to produce cDNA from all time points. To control for cDNA quantity, RT-PCR analysis was carried out for β-actin. All samples were normalised with respect to the β-actin control (Figure 4.2 (a)).

To ascertain when WT1 is first expressed during RA differentiation, semi-quantitative RT-PCR for WT1 was carried out. Primers for WT1, spanning from exon 4 to exon 6, were used to detect expression of WT1. The WT1 transcripts in this RT-PCR give two bands corresponding to the absence or presence of the alternative splice variant, exon 5. Figure 4.2 shows that expression of WT1 is first detected at 48 hours following RA differentiation. This expression continues culminating in high expression at 96 hours. In contrast, the expression of the POU transcription factor Oct-4 is down-regulated, with no expression detectable after 72 hours (Figure 4.2 (a)).



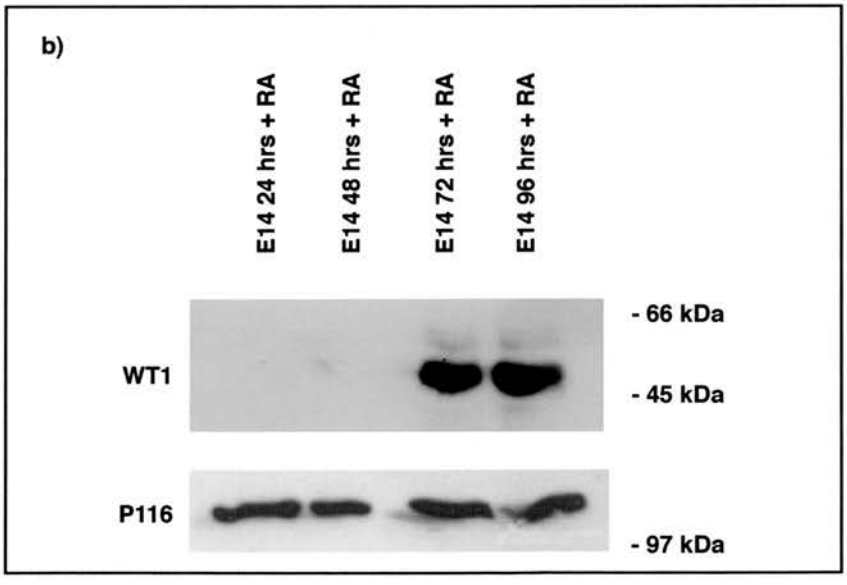


Figure 4.2 Analysis of the Kinetics of WT1 Induction in RA Treated ES Monolayer Cultures

a) 5 μg of total RNA was reversed transcribed to produce cDNA.To control for the quantity of cDNA, samples were normalised with respect to β-actin (25 cycles). The expression of WT1 mRNA was analysed by semi-quantitative RT-PCR using primers spanning exon 5 of WT1 (26 cycles). The expression of Sonic Hedgehog mRNA (26 cycles) was also analysed as it represents a direct target of the RA pathway, whilst Oct-4 was analysed to show the differentiation of the ES cells (27 cycles).

b) Expression of the WT1 protein was analysed by Western blot. 40 μg of nuclear protein from ES treated with RA for 24, 48, 72 and 96 hours, was separated on a 10% SDS PAGE gel and transfered onto nitrocellulose. The blot was probed for WT1 with the C-19 antibody. To control for loading the blot was stripped and probed for p116, a U5 small nuclear RNP-associated protein.

The induction profile of WT1 protein was analysed by collecting nuclear protein from RA differentiated ES cells at 24, 48, 72, 96 hours (Figure 4.2 (b)). 40 μg of nuclear protein was separated on a 10% SDS PAGE gel and transferred onto nitrocellulose. The C19 WT1 antibody was used to probe the blot for the presence of WT1. WT1 protein was detected after 72 hours of induction with RA. As a control for loading, the blot was stripped and reprobed with an antibody for P116, demonstrating equal loading of protein (Figure 4.2 (b)).

Analysis of the kinetics of WT1 induction in the ES monolayer suggests that the expression of WT1 is a result of either cellular differentiation or the emergence of a specific WT1 expressing cell lineage, rather than a direct transcriptional effect of RA. This is supported by analysis of the induction of Sonic Hedgehog (Shh), which has been demonstrated to be a direct transcriptional target of the retinoic acid pathway (Riddle *et al.*, 1993; Chang *et al.*, 1997). The expression of this transcript can be detected at the first time point taken 18 hours after the addition of RA (Figure 4.2 (a)). This is in contrast with WT1 expression, which is detected after 48 hours. Furthermore analysis of the murine WT1 promoter failed to detect the presence of a retinoic acid response elements (RAREs), which are present in the proximal promoters of RA target genes (Qian *et al.*, 2000), supporting the notion that WT1 is not a direct transcriptional target of the retinoic acid pathway.

4.2.2 Analysis of Lineages generated from RA

Having established that RA differentiation of ES cells leads to expression of WT1, we next carried out immunofluorescence for WT1 on differentiated ES cells following 96 hours of RA treatment. Using the C-19 antibody, a nuclear staining pattern for WT1 was detected in approximately 75% of differentiated ES cells (Figure 4.3 (a)). Next we investigated whether there was a specific lineage

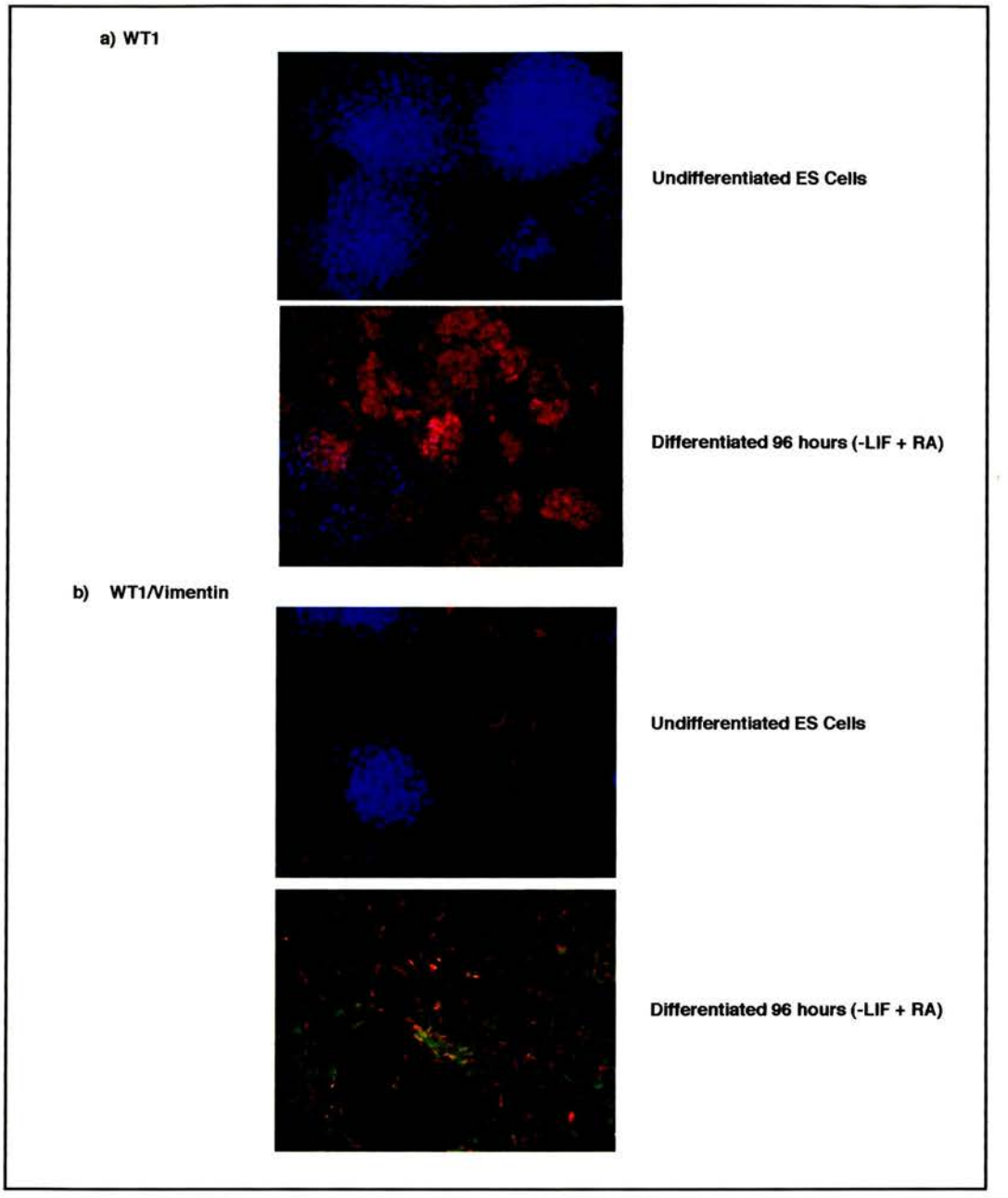


Figure 4.3 WT1 is Expressed in 80% of RA Treated ES Monolayer Cultures and WT1 Positive Cells Co-express Vimentin

a) Antibody staining was carried out for WT1 (red) on undifferentiated ES cells and ES cells treated RA for 96 hours. Cells were stained with dapi (blue) to visualise the nuclei. (Magnifcation x 20)

b) Co-staining for vimentin (red) and WT1 (green) was carried out on undifferentiated ES cells and ES cells treated with RA for 96 hours. (Magnification x 10)

within the RA differentiated monolayer system that was expressing WT1 by using immunofluorescence.

Given the restricted expression of WT1 *in vivo* to cells that possess mesenchymal properties, we used vimentin as a marker as it is expressed predominately in cells of mesenchymal origin, to see if WT1 expression was present in mesenchymal lineages. In the differentiated monolayer cultures, vimentin expression is high, with the majority of vimentin positive cells being also WT1 positive. This would suggest that mesenchymal cells are present in the RA differentiated monolayer which also express WT1 (Figure 4.3 (b)). To determine whether endothelial lineages were present, differentiated ES monolayer cultures were assayed for the expression of Flk-1. Staining of differentiated ES cells showed no detectable expression of Flk-1 (Data not shown).

To gain further insights into the lineages that were being expressed during the RA differentiation of ES cells, the expression of a variety of lineage and tissue specific transcripts was assayed. The expression of marker genes indicative of the three germ layers, mesoderm, endoderm, ectoderm and also some markers of specific lineages including neuronal, myogenic and haematopoietic were analysed by semi-quantitative RT-PCR. 5μg of RNA from the different time points of the RA differentiation protocol (0, 18, 24, 36, 48, 60, 72, 90, 96 hours) were reverse transcribed to produce cDNA. As a control for the RT-PCR and detection of specific transcripts, RNA from wild-type whole mouse embryos at E9.5 was prepared and reverse transcribed to produce cDNA. To control for cDNA quantity, RT-PCR analysis was carried out for β-actin, and all samples were normalised with respect to this control (β-actin) (Figure 4.2 (a)).

The transcripts chosen for mesodermal lineages were T-brachyury, which is expressed in early mesoderm, the myogenic regulatory specific transcripts, myf5 and myoD and finally β-globin, a definitive marker of blood. All three transcripts were detected in the cDNA sample generated from the E9.5 whole embryos.

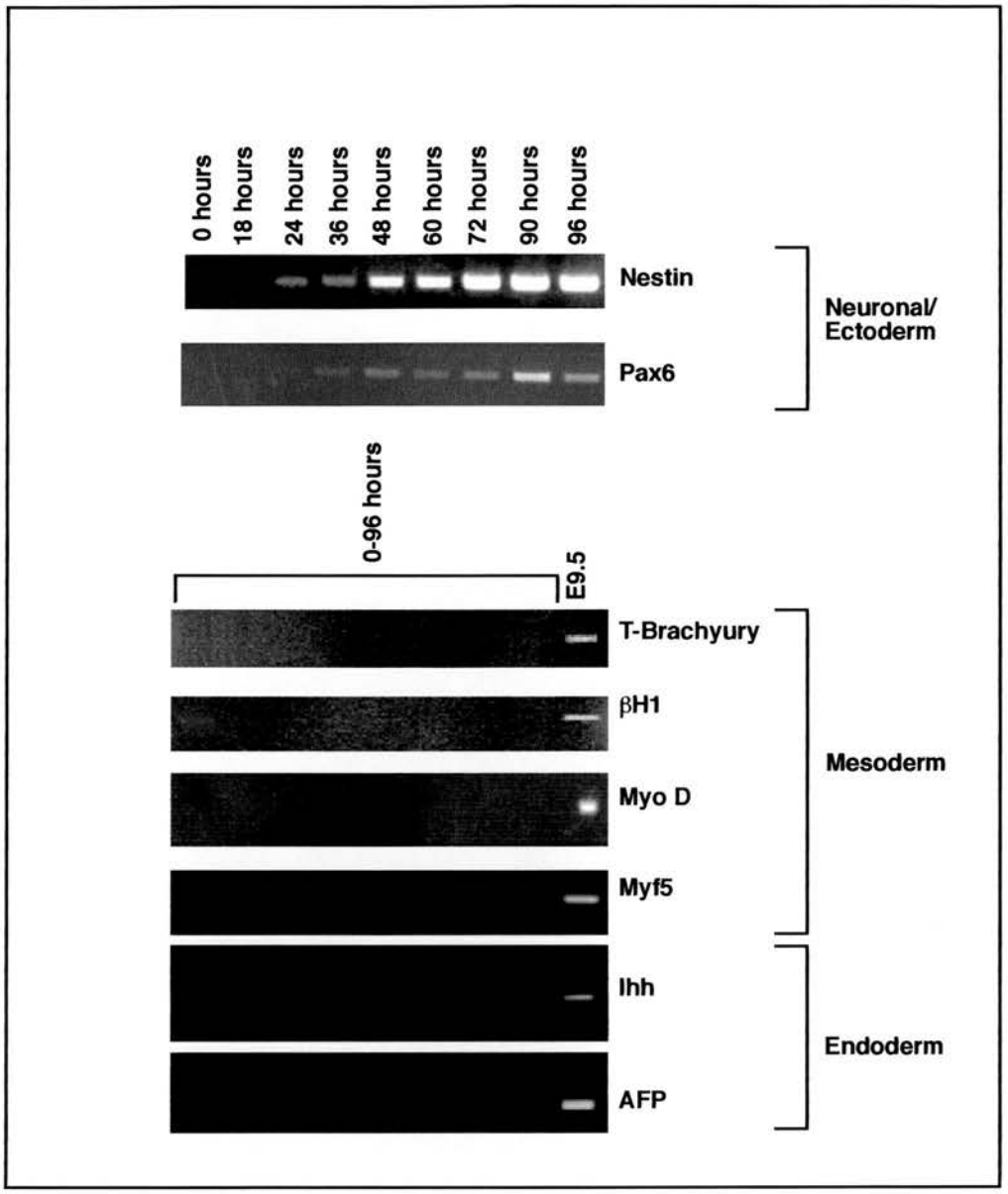


Figure 4.4 RA Treatment of ES Cell Monolayers leads to expression of Neuronal and Neuroectodermal lineage markers

Total RNA was collected from undifferentiated ES cells (0 hours) and ES cells treated with RA for 18, 24, 36, 48, 60, 72, 90 and 96 hours, and also E9.5 dpc wild-type whole embryos. 5 μg of total RNA was reverse transcribed to produce cDNA.To control for the levels of cDNA, RT-PCR for β-actin was carried out (26 cycles) and all samples were normalised with respect to this control.
The levels of various lineage specific transcripts were assayed by semi-quantitative RT-PCR. Nestin (27 cycles) and Pax-6 (27 cycles), for T-Brachyury, βH1, MyoD, Myf5, Ihh and AFP 35 cycles were carried out on the RNA from RA treated ES cells, whilst 26-28 cycles were used to detect the transcript in the RNA from the E9.5 embryos.

However there was no detection of these transcripts in the RA differentiated ES cells. Similarly there was no expression of alpha-fetoprotein or Indian Hedgehog, markers of endoderm. Analysis of Pax6, a marker for the neuroectodermal lineages, was detected in the cultures, as was the expression of nestin, a marker for neuronal progenitors (Figure 4.4).

Therefore, the analysis of a select panel of transcripts and antibodies, indicate that within the ES monolayer, there is an absence of mesodermal and endodermal lineages and presence of early neuronal and neuroectodermal lineages.

4.3 Expression of WT1 in Neuronal Progenitors

4.3.1 WT1 is Co-expressed with the Neuronal Stem Cell Marker Nestin

RT-PCR analysis demonstrates that within the monolayer system, there is expression of the early neuronal specific marker, nestin (Figure 4.4). In the original study by Scharnhorst *et al*, WT1 expression was present in subpopulations of RA differentiated EC cells that co-expressed GFAP (Scharnhorst *et al.*, 1997). The authors hypothesised that the co-expression of WT1 and the astrocyte specific marker GFAP may reflect the situation *in vivo*, given the reported expression of WT1 in a domain of the CNS (Armstrong *et al.*, 1993; Rackley *et al.*, 1993; Moore *et al.*, 1998). *In vivo*, nestin is an intermediate filament protein expressed during the development of the CNS and is a characteristic marker of CNS progenitor cells (Lendahl *et al.*, 1990). As the multipotential CNS progenitor cells terminally differentiate to become neurons and astrocyctes, nestin is down-regulated and replaced by expression of neurofilaments and glial fibrillary acidic protein (GFAP) respectively. A similar transition is observed in muscle development, in which nestin is transiently expressed in presomitic mesoderm and in striated cells of the myotome layer of the somites (Lendahl *et al.*, 1990). This expression is replaced by desmin in mature muscle. Nestin expression has also been detected in mesenchymal cells of

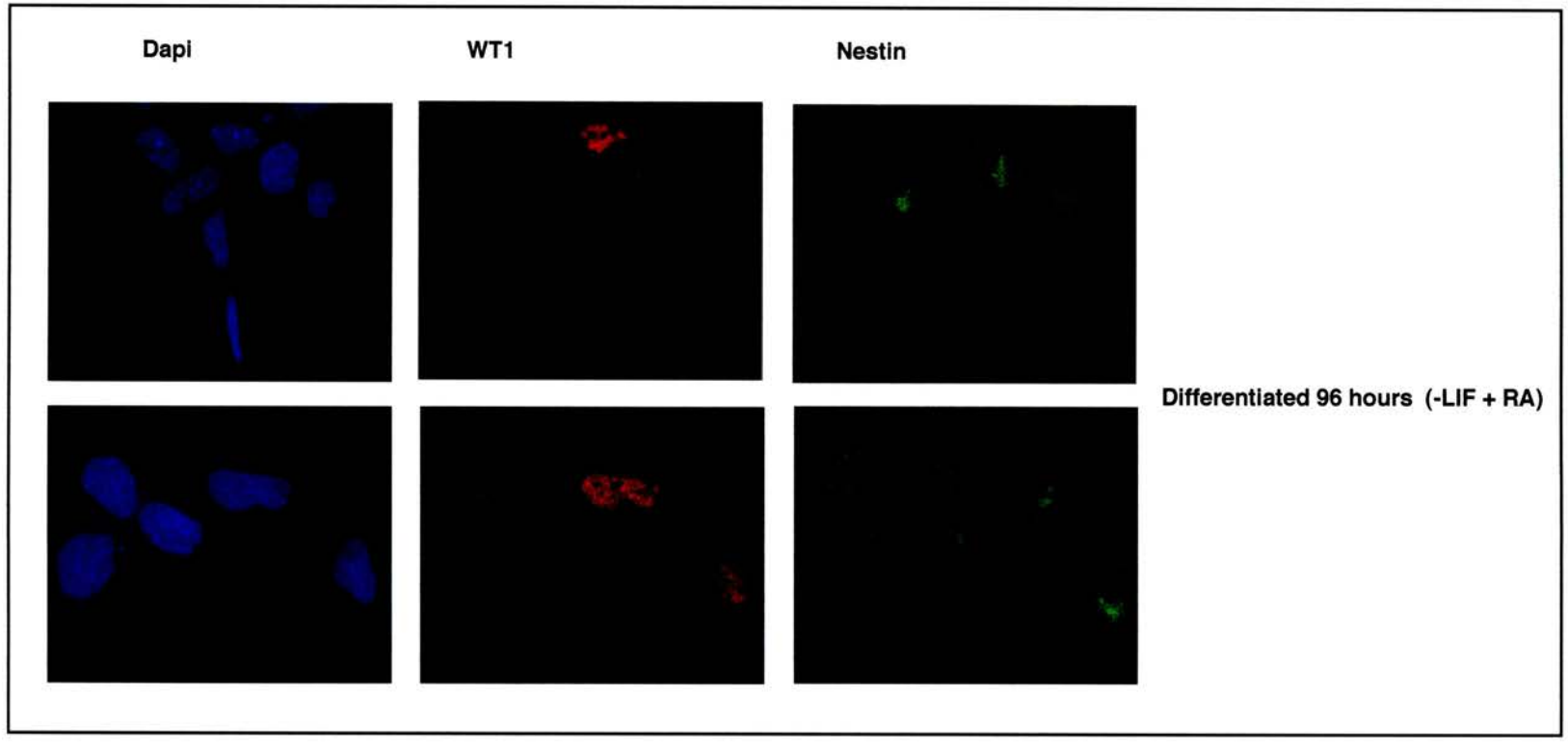


Figure 4.5 WT1 and Nestin are Co-expressed in RA Treated ES monolayer Cultures

Antibody staining for WT1 (red) and nestin (green) was carried out on RA differentiated ES cells after 96 hours. Cells were stained with Dapi to visualise the nuclei (Magnificaton x 10)

the developing pancreas (Selander and Edlund, 2002). However the absence of T-Brachyury, myoD and myF5, discounts the presence of mesodermal and/or muscle differentiation in the monolayer differentiation system and points to neuronal lineages being the probable cause of nestin expression.

Given the presence of nestin in the RA induced monolayer system, the question is whether a population of cells that possess characteristics of neuronal progenitors be expressing WT1? To answer this question, double immunofluorescence was performed using an antibody specific for nestin (Gift Dr. Meng Li) along with WT1. Monolayer cultures differentiated with RA for 96 hours were analysed for the co-expression of nestin and WT1. A subpopulation of cells that co-expressed nestin and WT1 were detected in the RA-differentiated system (Figure 4.5). However there were also cells that expressed WT1, but were nestin negative. This indicates that not all WT1 positive cells within the monolayer at 96 hours represent cells that have characteristics of neuronal progenitor cells. To determine whether these $WT1^{+ve}$ $nestin^{-ve}$ cells represented more mature neuronal cell types, which have down regulated nestin expression, the pan-neuronal markers, neurofilament 68 kDa and β-tubulin and the astrocyte specific marker GFAP were used in combination with WT1. Immunofluorescence with each of the markers showed no detectable expression at 96 hours, suggesting that $WT1^{+ve}$ $nestin^{-ve}$ cells are not mature neurons (Data not shown). This indicates that non-neuronal lineages are present in the differentiated cultures which also expresses WT1. The absence of GFAP staining in the differentiated ES cells is in contrast to the situation in EC cells differentiated with RA, in which $WT1^{+ve}$ $GFAP^{+ve}$ were detected (Scharnhorst *et al.*, 1997). However the EC cultures were differentiated for a longer period of time (6 days) and therefore may contain more differentiated neuronal lineages.

4.3.2 Expression of WT1 in Sox2 Restricted Neuronal Progenitors

The observation that WT1 was co-expressed in a sub-population of nestin positive differentiated ES cells leads to the possibility that WT1 may be

expressed in early neuronal progenitors. Therefore the analysis was extended to see whether WT1 expression was present in homogenous neuronal progenitors that were derived from two different ES cell differentiation protocols.

Expansion and fate choice of pluripotent ES stem cells *in vitro* along neuroectodermal lineages is regulated by a number of signals, for example retinoic acid and BMP signalling, which also act *in vivo* to control the proliferation and differentiation of CNS neural progenitor cells (For review see Panchision and McKay, 2002). However the generation of neuronal lineages from ES cells is highly variable and can lead to heterogenous cultures containing a variety of other lineages. Typically for the induction of neuronal differentiation, ES cells are aggregated to form EBs, exposed to neuronal inducing signals and then dissociated onto a permissive substrate which allows neuronal lineages to grow (Bain *et al.*, 1995; Renoncourt *et al.*, 1998). However the reliance upon aggregation, and thus the variation in structural organisation and positional information within the EBs during differentiation results in heterogeneity within EBs. Exposure of differentiating cells to potentially inappropriate signalling environments resulting from the juxtaposition of temporally and spatially distinct cell populations, results in various non-neuronal lineages being present in the EBs. Also the addition of growth factors to EBs can drastically affect the proportions of different cell types obtained in EB differentiation. In the absence of neuronal inducers, neurons represent a small percentage of cells. Efficient generation of neurons from EBs requires an additional stimulus. If that additional signal is retinoic acid, neuronal gene expression and neuronal development is activated, with the effect of repressing mesodermal gene expression (Bain *et al.*, 1996). Although there have been many modifications to this technique to increase the percentage of neurons, other lineages may still persist in the cultures.

In an attempt to isolate a homogenous pool of neuronal progenitors, ES cells in which a neomycin selection cassette was targeted into the Sox2 locus by

homologous recombination were produced (Li *et al.*, 1998). The Sox proteins constitute a family of transcription factors related to the mammalian testis-determining factor SRY through homology with their HMG-box DNA binding domains. Sox1 and Sox2 represent a subfamily of Sox genes (Sox1, Sox2 and Sox3), that are expressed within the developing nervous system. Sox2 is one of the earliest known transcription factors expressed in the developing neural tube, and is confined to the neuroepithelium of the neural plate and dividing neural progenitors in the early mouse embryo (Uwanogho *et al.*, 1995; Collignon *et al.*, 1996; Pevny *et al.*, 1998; Wood and Episkopou, 1999). Using this genetically modified ES cell line, it becomes possible to obtain a starting population of homogenous neuronal progenitors from a complex mass of cells following EB differentiation with retinoic acid and by exposing the cultures to G418. Sox2 expressing cells, which represent neuronal progenitors will survive, whilst the Sox2 negative non-neural cells in the differentiating cultures will be eliminated, because they lack G418 resistance.

Using this well characterised system we analysed the expression of WT1 at the 4 stages of the protocol via semi-quantitative RT-PCR. Using this system we can examine whether WT1 is expressed in pure neuronal progenitors (Figure 4.6 (a)). Total RNA was collected at the following stages of the differentiation pathway:-

(A) Day 4 (EBs differentiated in the absence of LIF for 4 days in suspension)

(B) Day 6 (EBs differentiated for 2 days in the presence of 1μM RA)

(C) Day 8 (EBs differentiated for 6 days in the presence of 1μM RA and G418)

(D) Day 10 (Cells dissociated and cultured in DMEM/F12 supplemented with N2)

2 μg of RNA was reverse transcribed to produce cDNA from the samples. To control for cDNA quantity, RT-PCR analysis was carried out for β-actin. All

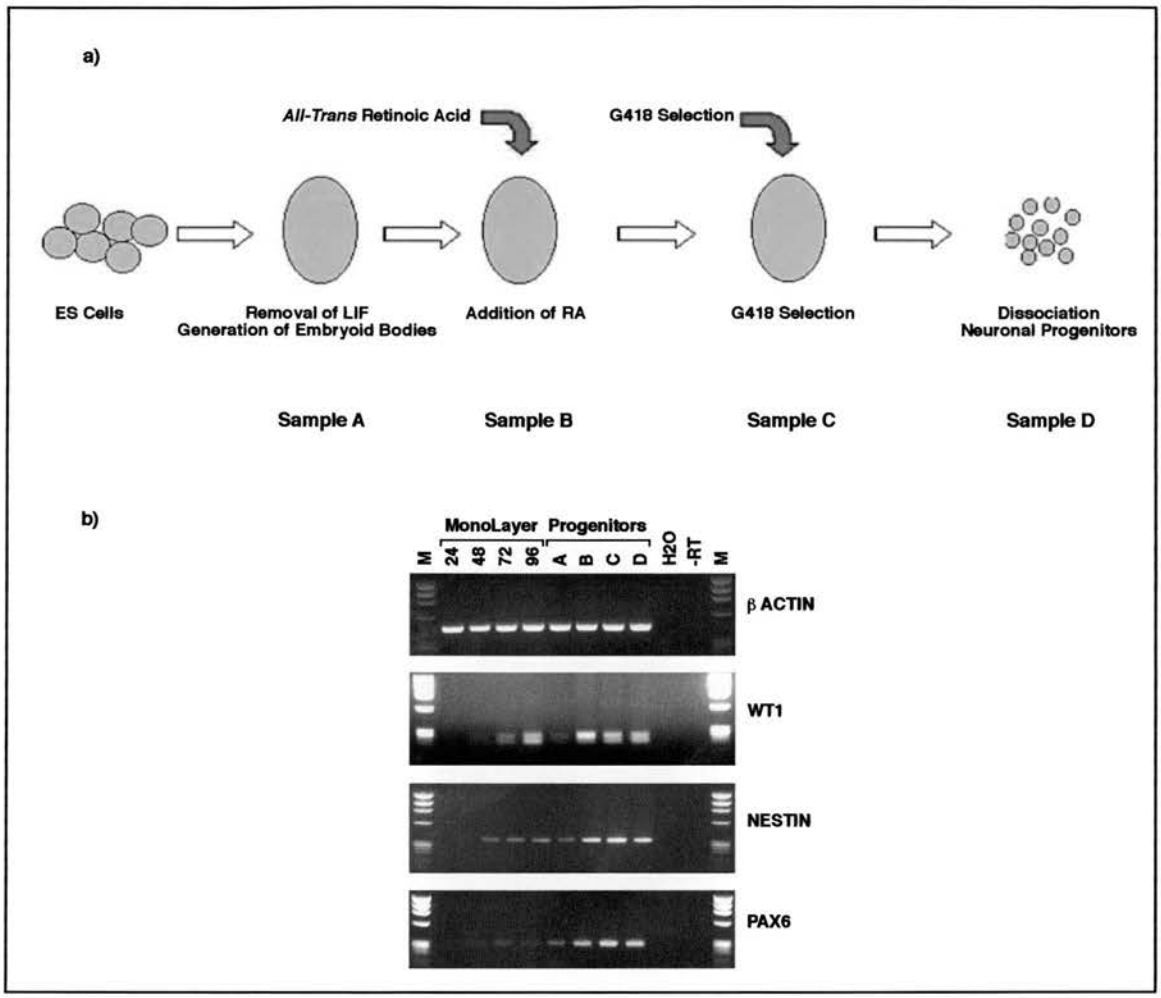


Figure 4.6 WT1 is Expressed in Purified Neuronal Precursors Derived from ES cells via Lineage Selection.

a) ES cells were targeted via homologous recombination with a IRES-G418 selection cassette into the SOX2 locus (Li *et al*, 1998). Total RNA was collected from the following stages: ES cells were allowed to aggregate in suspension culture in the absence of LIF for 4 days (Sample A). EBs were differentiated for a further 2 days in the presence of 1μM of RA (Sample B). EBs were differentiated for a further 2 days in the presence of G418 and 1μM RA (Sample C). EBs were dissociated and cultured in DMEM/F12 supplemented with N2 (Sample D).

b) 2μg of total RNA was reverse transcribed to produce cDNA. The expression of WT1, Nestin and Pax6 (26-28 cyles) were analysed via semi-quantitavtie RT-PCR. To control for cDNA quantity the samples were normalised with respect to β-actin (26 cyles). The expression of the transcripts in neuronal progenitors was compared to their expression in RA treated ES monolayer culture system.

samples were normalised with respect to the internal control (β-actin). RT-PCR was carried out for WT1 using primers that are specific for the exon 5 alternative splice site (Figure 4.6 (b)).

In sample A, there is low level expression of WT1. This sample represents RNA collected from day 4 EBs, which have been cultured in medium lacking LIF and allowed to aggregate and to form EBs. There is also low level expression of nestin and Pax6 in this sample.

The level of WT1 expression has increased dramatically in sample B, which represents RNA collected from day 6 EBs which have been differentiated for 4 days in the absence of LIF and a further 2 days in the presence of 1 μM *all-trans* retinoic acid. At this point within the EBs there will be heterogeneity, with the presence of non-neuronal lineages (Bain *et al.*, 1995; Li *et al.*, 1998).

WT1 is detected in sample C, but at lower levels than in sample B. Sample C represents RNA collected from day 8 of the differentiation protocol in which EBs have been through RA differentiation and G418 selection. The reduction of WT1 expression in sample C, in contrast to the levels of WT1 in sample B, may reflect the removal of the non-neuronal cell types, which also expressed WT1. Within this pool of cells there is expression of neuronal progenitor subtypes markers, including Pax6, which *in vivo* is expressed in the dividing neural precursors throughout the length of the embryonic tube, and the LIM homeodomain protein Islet1, an early marker of motor neuron differentiation.

RNA from sample D was collected from day 10 of the differentiation protocol. At this stage the EBs have been through G418 selection to remove non-neuronal lineages, dissociated, plated and grown in a defined culture medium of DMEM/F12 plus N2 supplement. At this point in the protocol, 95% of the cells express Sox1 and Sox2, with the neuronal progenitors exhibiting small, ovoid

morphology typical of neuroepithelial cells *in vivo* (Li *et al.*, 1998). There is expression of WT1 in this homogenous pool of neuronal progenitors.

4.3.3 Absence of WT1 Expression in Non-RA ES Derived Neuronal Progenitors

We also analysed the expression of WT1 by RT-PCR in ES derived neuronal progenitors which have been isolated on the basis of nestin expression (Lee *et al.*, 2000). The differentiation process involves 4 steps: generation of EBs (Step2), use of a defined medium to select for nestin positive CNS progenitors (Step3), the proliferation of nestin positive CNS progenitors in the presence of a mitogen (Step4) and the differentiation of the CNS progenitors by the removal of the mitogen (Step5). The protocol does not employ the use of retinoic acid as an inducer for neuronal differentiation. Instead the ES cells are allowed to aggregate in the absence of LIF to form embryoid bodies, dissociated and then grown in ITSFn medium (Insulin, transfferin, selenium and fibronectin) (Okabe *et al.*, 1996; Lee *et al.*, 2000). In the presence of this defined medium, nestin positive cells are produced, which are subsequently selected for further expansion. The nestin positive cells are then differentiated to produce functional neurons (Figure 4.7 (a)).

5μg of RNA was obtained from stages 3, 4 and 5 of the differentiation protocol (Gift Dr. R.D McKay). The 5 μg of RNA was reverse transcribed to produce cDNA from the sample. To control for cDNA quantity, RT-PCR analysis was carried out for β-actin. All samples were normalised with respect to the internal control (β-actin). RT-PCR was carried out for WT1 using primers that are specific for the exon 5 alternative splice site.

There was no detection of WT1 expression in the RNA collected from stages 3, 4 and 5. As a control, cDNA from sample C of the Sox2 neuronal differentiation protocol was included in the RT-PCR analysis. Expression for WT1 is detected

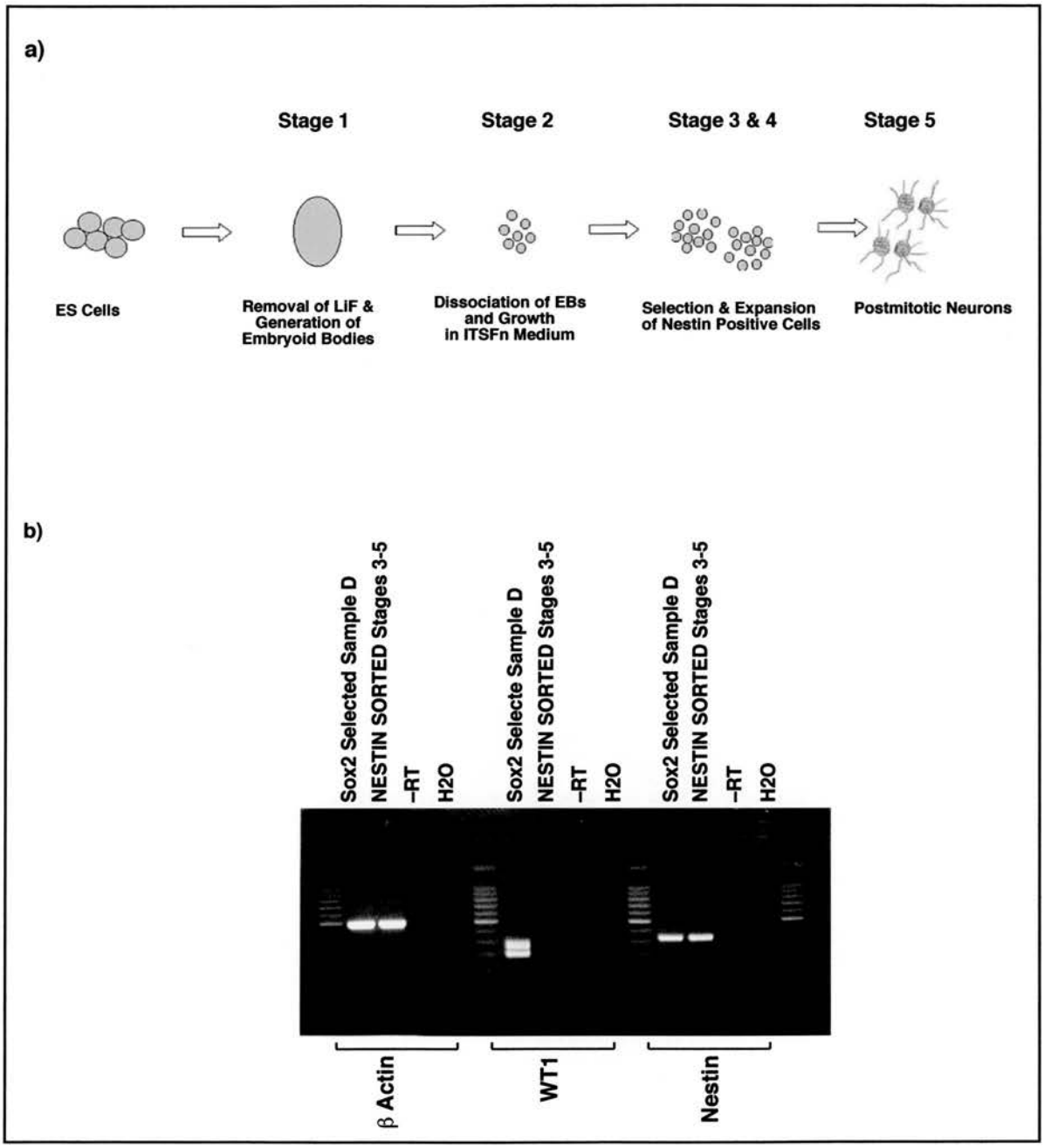


Figure 4.7 WT1 is Not Expressed in Neuronal Progenitors Derived From ES Cells Selected by the Expression of Nestin

a) Diagram showing the differentiation protocol used to derive neuonal progenitors from ES cells. Total RNA was collected from stages 3-5 of the differentiation protocol

b) 5μg of total RNA from stages 3-5 was reverse transcribed to produce cDNA. The expression levels of WT1 and nestin were assayed by semi-quantitative RT-PCR(26-28 cycles). To control for cDNA quantity the samples were normalised with respect to the expression levels of β-Actin (26 cycles). The expression of WT1 in the nestin purified neuronal progenitors and post-mitotic neurons was compared with Sox2 selected neuronal progenitors.

in the Sox2 sample. In both samples there is expression of nestin at similar levels (Figure 4.7 (b)).

4.4 Expression of WT1 in the Developing CNS

Given the expression of WT1 in neuronal progenitors derived from the Sox2 selected ES cells, could WT1 be expressed in neural progenitors *in vivo*? Within the developing nervous system, several studies have reported expression of WT1. Sites of expression include discrete pockets of staining in the developing eye, spinal cord and in the ventricles of the brain (Armstrong *et al.*, 1993; Rackely *et al.*, 1993; Moore *et al.*, 1998). Apart from in the eye, where the function of WT1 has been described and a phenotype observed, no other studies have indicated what neuronal cell type WT1 is expressed in, or have proposed a function for WT1 expression in the CNS. Therefore we set about mapping the expression of WT1 in the early stages of neuronal development spanning E9.5-E10.5.

4.4.1 Expression of WT1 in the Ventral Neural Tube

The WT1 mouse monoclonal antibody (H2) was used to localise WT1 expression in E9.5 and E10.5 embryos. In transverse sections of E9.5 embryos, there was no detectable expression of WT1 in the developing CNS. There was expression of WT1 detected in the pronephric/mesonephric ridge (Data not shown). At E10.5, expression of WT1 was evident in two populations of cells in the ventral half of the neural tube (Figure 4.8). This expression of WT1 was detected at a similar ventral position throughout the length of the neural tube, from cervical regions to a posterior limit of expression in the hindlimbs. There were no other sites of WT1 expression within the CNS at E10.5. WT1 staining was detected in the mesonephric ridge and also the mesothelium lining of the thoracic cavity (Figure 4.8 (a) and (b)). As a control, sections were processed in which primary antibody was absent. In these sections there is no staining in either the neural tube or the mesonephric ridge at E10.5 (Figure 4.8 (c)). This

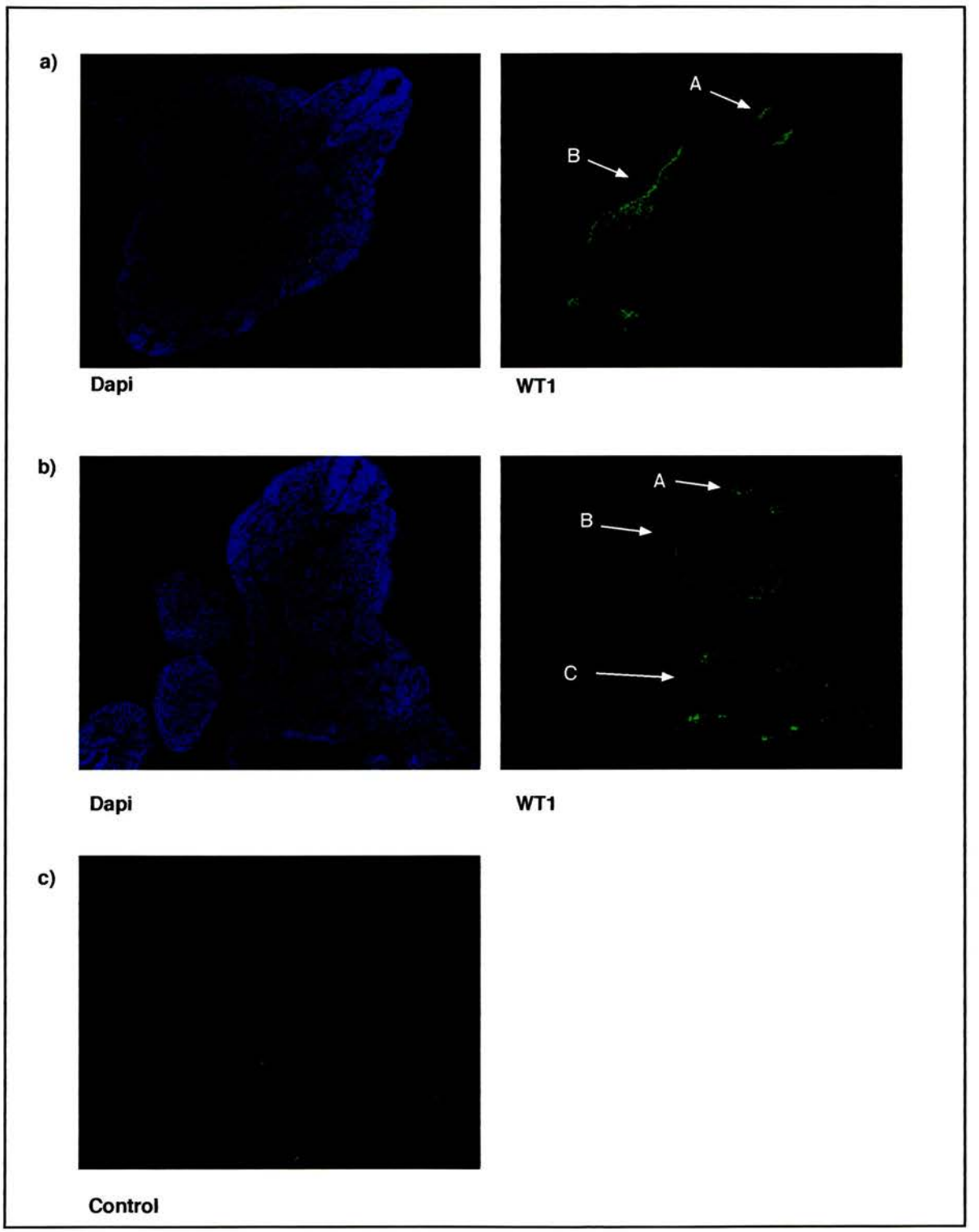


Figure 4.8 WT1 is Expressed in the Ventral Neural Tube

10 μM transverse sections of E10.5 embryos were stained with WT1 (green). At the level of the (a) branchial and (b) lumbar region, WT1 can be detected in the ventral half of the neural tube. As a control, sections from the brachial region were processed without the addition of the primary antibody (c). The sections were stained with dapi (blue) to visualise the nuclei. (A) neural tube (B) mesonephric ridge (C) mesothelium (Magnification x 20).

endogenous staining is consistent with the findings from the human WT1 YAC LacZ mice (Moore *et al.*, 1998).

4.4.2 WT1 Expression Partially Overlaps with Nkx6.1 Expression and with Islet 1

With the detection of WT1 in the ventral neural tube at E10.5, the next step was to map the expression of WT1 in relation to the well-defined ventral neural subtypes. These have been established from the expression profile of several transcription factors (For review see Briscoe & Ericson, 2001). During ventral neural tube patterning the morphogen, Sonic Hedgehog (Shh), is secreted from the notochord to regulate the regional expression of several different transcription factors (Marti *et al.*, 1995). Recent studies have characterised a family of transcription factors that delineate five neuronal progenitor cell domains in the ventricular zone of the ventral neural tube, referred to as p0, p1, p2, pMN and p3 (For review see Jessell, 2000; Briscoe & Ericson, 2001). Using lineage tracing experiments and genetic markers, it has been demonstrated that distinct classes of cells arise from specific progenitor cell domains positioned along the dorsal/ventral axis of the ventricular zone (For review see Jessell, 2000). Cell-fate specification is thought to occur through the initiation of complex transcriptional cascades that activate appropriate neuronal subtype determining genes, whilst simultaneously suppressing alternative pathways of differentiation. Once determined, these cells exit the cell cycle in a regulated manner and generate postmitotic neurons. These postmitotic neurons then migrate to defined positions within the neural tube. Initial migration of newly generated neurons occurs in a medio-lateral plane, i.e. more lateral the position of a neuron, the more differentiated. Based on this V0-V3 interneurons develop from the p0-p3 domains, respectively, and motor neurons (MNs) arise from the pMN domain.

The three most ventral progenitor domains, p3, pMN and p2 are established by the combinatorial activities of three homeodomain proteins, Nkx6.1, Nkx2.2 and

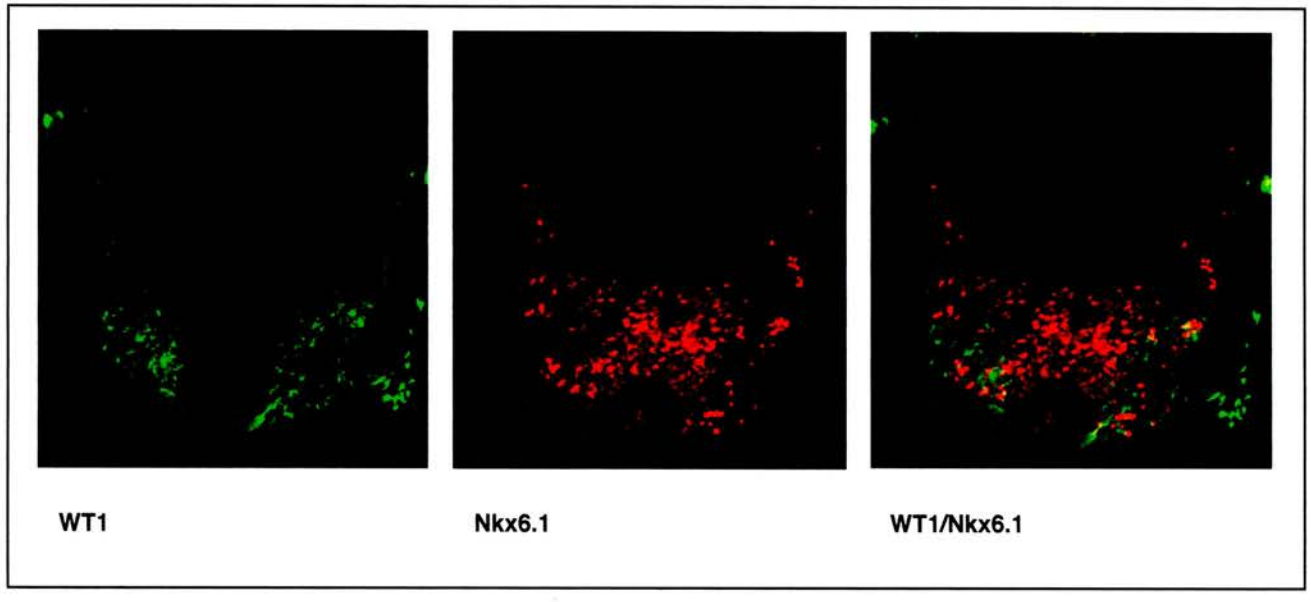
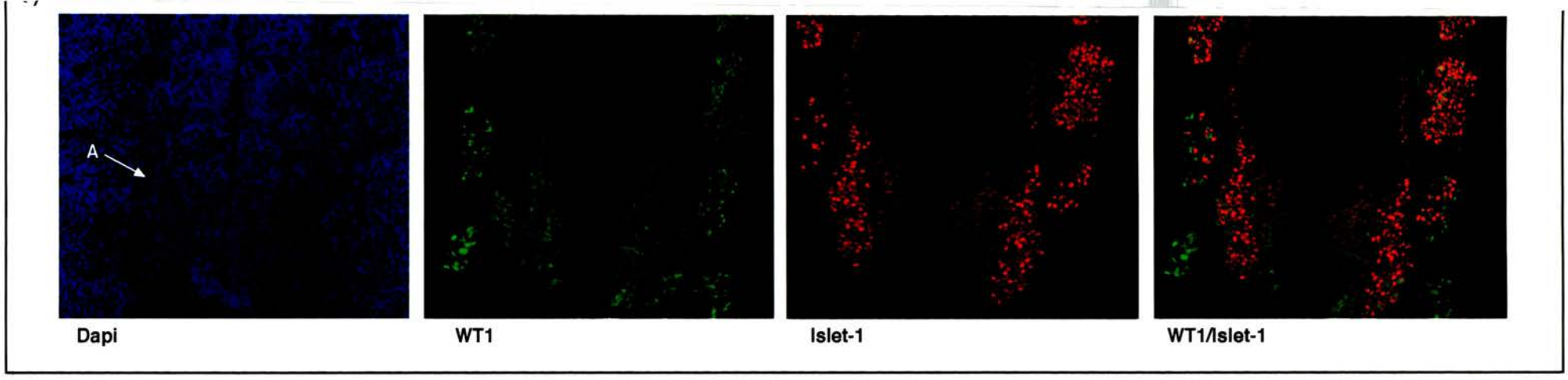


Figure 4.9 WT1 Expression in the Ventral Neural Tube Partially Overlaps with Nkx6.1

10 μM transverse sections from the brachial level of E10.5 neural tube were co-stained with WT1 (green) and Nkx6.1 (red) (Magnification x 40)



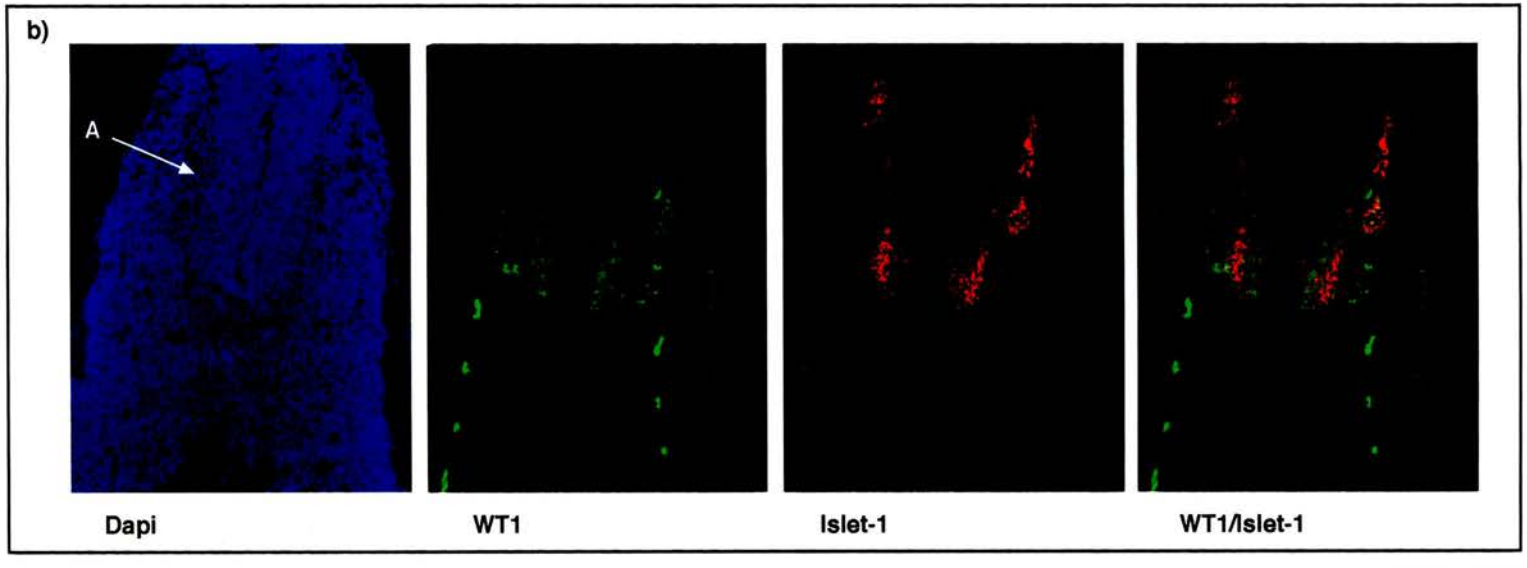


Figure 4.10 WT1 Expression in the Ventral Neural Tube Partially Overlaps with Islet-1

10 μM transverse sections from the branchial (a) (Magnification x 63) and the lumbar (b) (Magnification x 40) regions of E10.5 neural tube were co-stained with WT1 (green) and the motor neuron marker, Islet-1 (red). Sections were stained with dapi (blue) to visualise nuclei. (A) Nerual Tube.

Irx3 (For review Briscoe & Ericson, 2001). Since Nkx6.1 marks these three progenitor domains, a rabbit Nkx6.1 polyclonal (Gift Dr P.Serup) was used alongside the WT1 monoclonal antibody on transverse sections of E10.5 neural tube (Figure 4.9). In comparison to the expression profile of Nkx6.1, which is expressed from the mid-line in a broad ventral domain, WT1 is expressed more laterally in comparison. However there is an overlap of WT1 staining with that of Nkx6.1. This indicates that WT1 is expressed in domains where neuronal progenitors are generated.

We next analysed the expression WT1 in comparison to the expression profile of Islet1 (Isl-1). Isl-1 is the first indicator of motorneuron differentiation and is expressed by all classes of motor neurons, preceding that of other LIM homeobox genes, thus defining an early and common step in motor neuron differentiation (Pfaff *et al.*, 1996). Double immunofluorescence analysis was performed for WT1 and Isl-1 using a rabbit polyclonal antibody (Gift Dr T.Jessell). At E10.5 strong expression of Isl-1 was evident in two bilaterally symmetrical populations of cells in the ventral neural tube. This expression profile represents the postmitotic motorneuron domain (MN domain). Co-staining for WT1 shows that the majority of the WT1 positive cells also co-express Isl-1, indicating that WT1 is expressed in motor neuron populations (Figure 4.10). However there is also expression of WT1 in a more medial domain, encompassing the Nkx6.1 domain, and also dorsally, which would indicate expression in the p2/V2 domains (Figure 4.10).

4.5 Discussion

The work presented in this chapter demonstrates that WT1 expression is induced in ES monolayers differentiated with RA. From the observed kinetics of WT1 induction in the system, it is unlikely that the expression of WT1 is a direct result of retinoic acid signalling pathways. This suggests that the induction of WT1 is the result of the emergence of a specific WT1 expressing lineage.

Using a small number of markers, either by immunofluoresence or RT-PCR analysis, analysis of the differentiated cultures indicates that the predominant lineages present in the culture are mainly neuroectodermal. The absence of mesodermal lineages in the RA differentiated ES cells is in agreement with the findings of Bain *et al* who demonstrated that RA treatment of embryoid bodies, resulted in repression of mesodermal lineages, including T-brachyury, and activation of specific neuronal transcripts (Bain *et al.*, 1996). However there is co-expression of WT1 and vimentin in the RA differentiated monolayer. Although predominately expressed in mesenchymal lineages, vimentin is also expressed in parietal endoderm cells, primary mesenchymal cells that delaminate from embryonic ectoderm and neural crest (Lane *et al.*, 1983; Franke *et al.*, 1982; Cochard and Paulin, 1984). Vimentin is also expressed by neural progenitors in the neuroepithelium of the CNS, where its expression is concurrent with that of nestin (Frederiksen and McKay, 1988). Co-staining of the cultures with WT1 and nestin demonstrates that there are populations of cells that express both markers, indicating that WT1 is expressed in differentiated ES cells that possess characteristics of neuronal progenitors. This suggests that the vimentin staining in not indicative of mesenchymal cells populations but due to the presence of neuronal progenitors or neuroectodermal lineages. Although early neuronal markers are present, there is an absence of expression of more differentiated neuronal markers in the cultures. Also there are populations of WT1 positive cells within the monolayer system, which do not express the specific neuronal makers, indicating that other non-neuronal WT1 expressing lineages are present.

From the *in vitro* analysis of ES derived neuronal progenitors, WT1 is detected in neuronal progenitors, but only present in the system using RA as an inducing agent. Why is there a difference in expression of WT1 in the two systems employed to generate ES cell derived neuronal progenitors, when both starting progenitor populations express the neuronal stem cells marker nestin? One

possible explanation for the difference is that the two systems are producing different types of neuronal progenitors.

In investigating which CNS type the non-RA induced ES cell derived neuronal progenitors represent *in vivo*, expression of three position specific markers along the anterior-posterior axis was analysed in the cultures (Okabe *et al.*, 1996; Lee *et al.*, 2000). Analysis of the stages in the protocol by RT-PCR shows that there is high expression of OTX-1 and En-1 in stages 2-4 (Lee *et al.*, 2000). OTX-1 is expressed during the early stages of neuroectoderm differentiation, whilst engrailed (En-1) is expressed in the midbrain/hindbrain boundary (Davis and Joyner, 1988; Simeone *et al.*, 1998). However expression of Hoxa-7, a marker of cells in the posterior spinal cord, is not detected in the neuronal progenitors derived from this differentiation system (Okabe *et al.*, 1996). Taken together the expression of these genes is indicative of early midbrain/hindbrain identity indicating the neuronal progenitors and lineages produced by this protocol have characteristics *in vivo* of midbrain and hindbrain neurons (Lee *et al.*, 2000).

In the RA induced ES derived neuronal progenitors, there is a shift in the type of neuronal progenitor generated in this system. Neural progenitors formed from EBs exposed to RA for 3 days, have been shown to lack expression of the early midbrain markers OTX-2 and En-1, and instead show expression of HoxC5, HoxC6 and HoxC8, markers of spinal cord identity (Wichterle *et al.*, 2002). The addition of RA to EBs to promote neuronal differentiation has been shown to repress the differentiation of midbrain dopaminergic neuronal differentiation (Kawasaki *et al.*, 2000; Wichterle *et al.*, 2002) and to lead to differentiation of progenitors towards ventral CNS progenitors (Renoncourt *et al.*, 1998; Wichterle *et al.*, 2002). This shift towards ventral CNS progenitors could be a result of RA directly activating Shh transcription (Riddle *et al.*, 1993; Chang *et al.*, 1997). In the developing ventral neural tube, Shh mediated signals from the notochord and floor plate, play an essential role in the differentiation of ventral neuronal progenitor subtypes. Shh signalling controls ventral neural fates by regulating the

expression of several transcription factors, including the Pax genes. *In vivo*, Pax6 is expressed in ventral progenitors of the neural tube (Ericson *et al.*, 1997). This suggests that the expression of WT1 in the RA induced Sox2 system may be indicative of WT1 expression in CNS progenitors found in the ventral neural tube.

In vivo, WT1 appears to follow this pattern of expression in the early developing CNS. At E10.5, there was no detection of WT1 in the developing brain, whilst expression was detected in the neural tube. Within the neural tube, WT1 expression was observed in the ventral half in a medial population of cells that represented neuronal progenitors, as determined by the expression pattern of Nkx6.1. However detection of WT1 can also be found in the motor neuron component of the neural tube, overlapping partially with that of Isl-1, but also dorsally in an expression domain that may represent the V2 inter neurons. The expression pattern of WT1 appears to follow that of the Lim homeodomain gene, Lim3 (Pfaff *et al.*, 1996, Thaler *et al.*, 2002). Lim3 is expressed throughout the neural tube and is involved in the generation of two adjacent, but distinct cell types, motor neurons and V2 inter neurons. Lim3 is transiently expressed in neuronal progenitors that give rise to motor neurons and also to V2 interneurons (pMN and p2) as these progenitors exit from the cell cycle and become post-mitotic neurons.

The expression of WT1 in a population of neuronal progenitors in the neural tube adds to the list of tissues in which WT1 has a been detected in progenitor populations. For example, WT1 expression has been described in progenitor populations in the heart, kidney, gonad and haematopoetic system. In the epicardium of the heart WT1 expression is highest in undifferentiated progenitors, and then becomes down regulated as the progenitors begin to express lineage specific markers. In the neuronal tube, WT1 expression is detected in a domains that mark neuronal progenitors, but its expression persists in differentiated post-mitotic neurons.

Mounting evidence suggests that WT1 has role in the development of some neuronal lineages. WT1 has been demonstrated to be critical for normal retina formation with a role in the proliferation of retinal progenitors and ganglia cell development (Wagner *et al.*, 2001). Recently it has been demonstrated that in an *in vitro* retinoblastoma tumour cell line, Y-79, WT1 was associated with the conversion of the undifferentiated Y-79 cells to neurons (Wagner *et al.*, 2002). However what is the function of WT1 in the neural tube? To date, no phenotype has been reported in the WT1 null mice involving patterning and generation of the neural tube subtypes. A preliminary screen in WT1 null mice at E10.5 demonstrates that Isl-1 positive cells are still present at similar levels compared to wild-type and heterozygote litter mates (data not shown). This therefore suggests that WT1 expression is not crucial for the generation of motor neurons.

Recently it has been demonstrated that developmentally relevant signalling factors can induce ES cells to differentiate into spinal progenitor cells, and subsequently into motor neurons through a pathway that recapitulates the developmental process *in vivo* (Wichterle *et al.*, 2002). From the observed expression of WT1 in motor neurons, this system would be highly relevant to study the role of WT1 in the generation of motor neurons. However a more suitable system to use would be WT1 loss-of-function experiments using the conditional *loxP* mice described in chapter 3 and specific neuronal *Cre* expressing mice. Currently several *Cre* expressing lines are published that would be suitable to dissect the role of WT1 in neuronal development. To specifically question the role of WT1 in motor neuron development, a *Cre* line under the control of Olig2 would be the most appropriate. Olig2 is expressed selectively in progenitor cells within the pMN domain and is involved in the differentiation of motor neurons (Novitch *et al.*, 2001). A mouse model, in which an inducible form of *Cre* has been knocked into the endogenous Olig2 locus, has been generated (Takebayashi *et al.*, 2002). Also recently Dr. Colin Miles has identified a 350bp regulatory element located 5kb upstream of the WT1 start site

that drives a LacZ reporter in the developing spinal cord. This fragment was identified by its high conservation between fugu, mouse and human. A construct containing the 350 bp fragment linked to a minimal Hsp68 promoter was used to produce transgenic mice that were analysed at mid-gestation (E12.5). Analysis of the offspring demonstrated that LacZ is detected in the ventral half of the neural tube (Dr Colin Miles, personal communication). It is intriguing to note that this regulatory element has been conserved from fish to mammals throughout evolution and thus may be of functional significance. This regulatory element would therefore be ideal for directing expression of *Cre* for WT1 loss-of-function studies.

Chapter 5 Generation of WT1 Null Embryonic Stem Cells

Chapter 5 Generation of WT1 Null Embryonic Stem Cells

5.1 Introduction

The embryonic lethality of the WT1 knockout mice has made it difficult to study the potential functions of WT1 in development. Establishing a conditional knockout of the *WT1* gene is one approach to overcome this issue, as described in Chapter 3. In parallel with the development of a conditional knockout, an alternative and complementary approach to study its function, is to establish WT1 null ES cell lines. These targeted embryonic stem cells can be utilized as an *in vitro* differentiation system to identify candidate WT1 target genes, and also to study the effect of WT1 loss-of-function on cellular differentiation pathways.

The use of an *in vitro* ES cell differentiation system has advantages over immortalised cell lines in that they are not restricted to particular differentiation processes. ES cells retain the ability to generate multiple lineages and during the course of differentiation, express tissue specific proteins, ion channels and receptors in a pattern closely resembling the pattern observed *in vivo*. In Chapter 4, the addition of RA to ES monolayer cultures has been shown to result in high-level expression of WT1. The use of this RA differentiation protocol provides an ideal system in which to investigate the effects of WT1 loss-of-function on gene expression.

A similar approach has already been employed, and has identified IGFBP-4 and cyclin G1 as potential WT1 target genes (Wagner *et al.*, 2001). Using gene targeting, ES cells were generated that were homozygous for point mutations in the WT1 zinc fingers (DDS ES cells), commonly found in Deny-Drash Syndrome patients. This modification leads to a truncation of WT1 protein in zinc finger 3, disrupting the DNA binding region (Patek *et al.*, 1999). Wild-type

and DDS ES cell lines were differentiated with RA, and the gene expression profiles of the two cell lines compared using cDNA macroarrays. The aim of this work is to analyse the effects of loss-of-function of WT1 on gene expression profiles in the RA differentiation system using Affymetrix Genechip arrays.

5.1.1 Use of Microarrays

Until recently, comparing expression levels across different tissues or cells was limited to tracking one or a few genes at a time. With the advent of microarray technology, it as now become possible to obtain quantitative information regarding the complete transcription profile of a cell or tissue at any given time. There are numerous examples where microarrays have answered several important biological and developmental questions. For example, microarrays have been successfully used to decipher the complex transcriptional regulatory networks involved in the developmental process of metamorphosis in *Drosophila* (White *et al.*, 1999). Metamorphosis is characterised by diverse developmental phenomena, including cellular proliferation, tissue remodelling, cell migration and programmed cell death. This developmental switch relies upon the integration of a set of processes that are controlled by a transcriptional hierarchy, which in turn co-ordinates the expression of hundreds of genes.

In *Drosophila*, metamorphosis is initiated in response to the hormone ecdysone, which activates this transcriptional hierarchy. Using microarrays, the transcriptional profile of gene expression of wild-type *Drosophila* during early metamorphosis was assayed at stages spanning two pulses of ecdysone. Changes in metabolism, myogenesis, neuronal and cellular differentiation were all observed in response to ecdysone (White *et al.*, 1999). The use of microarrays to study this developmental phenomenon allowed the authors to connect known signal transduction pathways to the ecdysone-initiated metamorphosis. It also assigned known genes to developmental pathways

associated with metamorphosis, and also identified genes not previously associated with this process.

Microarrays have also been also employed successfully to identify genes whose expression depends upon the function of a specific component of a transcriptional network. The paired-type homeobox transcription factor, CRX, has a pivotal role in the terminal differentiation of vertebrate photoreceptors (Furukawa *et al.*, 1997). Mutations in the human CRX gene result in either congenital blindness or photoreceptor degeneration (Swain *et al.*, 1997). Targeted mutation of the mouse CRX results in failure of development of the light detecting outer segment of photoreceptors (Furukawa *et al.*, 1999). Using microarrays, the transcriptional networks controlled by CRX in the developing retinal tissue from $CRX^{+/+}$ and $CRX^{-/-}$ mice were established (Livesey *et al.*, 2000). From this study, the authors identified a novel set of photoreceptor genes controlled by CRX, which function in a number of different stages in photoreceptor differentiation.

Recently, microarrays have been employed to produce a transcriptional profile that relates to the unique properties of stem cells. To date the list of genes known to be crucial for stem cell function, and which are used in the purification and isolation of stem cells, is relatively small. Therefore, a list of genes unique to stem cells would permit a greater understanding of the special properties of stem cells in their basic biology and also their potential clinical use. Microarray analysis of mouse embryonic, neural, haematopoietic stem cells and human haematopoietic stem cells by two independent groups generated a list of approximately 200 genes that were shared by these various stem cell populations (Ramalho-Santos *et al.*, 2002; Ivanova *et al.*, 2002). This list of transcripts enriched in all stem cell populations may include genes that are crucial for unique properties of the stem cell i.e. self-renewal and the ability to generate multi-lineage differentiated progeny. However a comparison of the lists for stem cell enriched transcripts showed only 15 genes that were shared

between the two studies. Despite this relatively low number, these studies provide an insight into the transcriptional networks that are present in the various stem cell populations analysed. What is evident is that stem cells from each compartment/region are distinct, in that each stem cell type can be identified by highly enriched genes that are not present (or enriched) in other stem cells.

The work presented in this chapter compares the gene expression profile of wild-type and WT1 null ES cells in the RA differentiation protocol using Affymetrix Genechip arrays. The outcome of this study may lead to the identification of novel candidate WT1 regulated genes.

5.2 Generation of WT1 Null Embryonic Stem Cells

The strategy chosen to generate WT1 null ES cells is to apply the same principle of *Cre-LoxP* conditional inactivation described in Chapter 3. From the first round of gene targeting, one allele has been created which is lacking exon 1 of *WT1*, producing a WT1 heterozygote null ES cell. The next step is to use the same targeting construct, *pWT1LoxP24*, and target the second remaining wild-type allele. This approach would produce an ES cell clone in which one *WT1* allele would be null, whilst the other would be a conditionally targeted allele, with *loxP* sites flanking exon 1. The system would permit temporal control over the ablation of WT1 during the differentiation of ES cells by the administration of *Cre*-recombinase. An example of how the system could be used is shown in figure 5.1.

5.2.1 Targeting Using *pWT1Lox*P24 Construct

Following the transient transfection of the homologous targeted parental clone, 14.17 with *pMC-Cre*, clones were selected in which type II recombination had occurred (removal of the full genomic interval between the 3 *loxP* sites, which includes exon 1 and the selection cassette). One clone, 14.17.3, which was

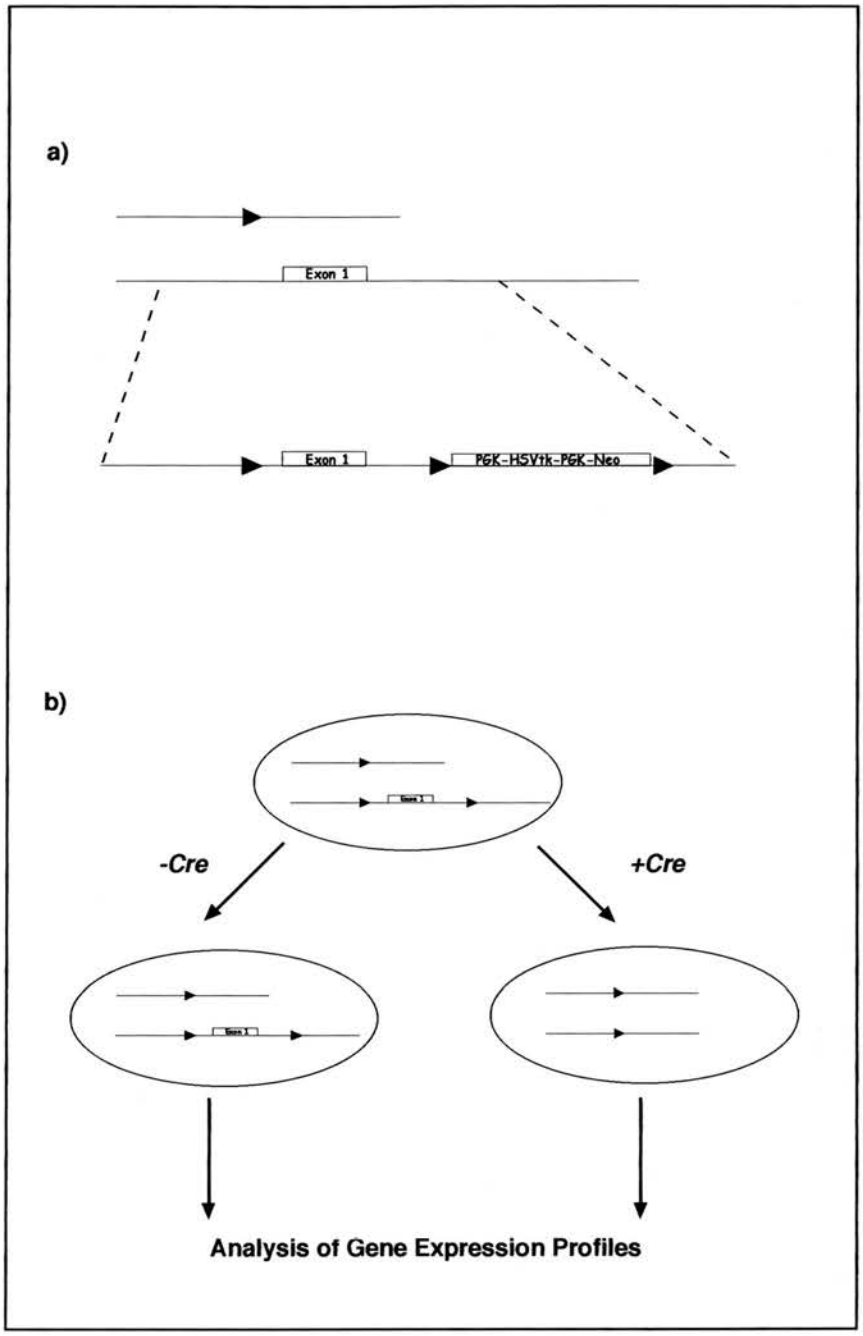


Figure 5.1 Generation and *In Vitro* Application of Conditional WT1 Null ES Cell Lines

a) Diagram showing targeting of the remaining wild-type *WT1* allele of the heterozygous null ES cell with the *pWT1LoxP* construct to create ES cells heterozygous for *WT1* in which induction of *Cre* expression will ablate WT1.

b) Individual clones of ES cells generated in this way can be used to compare gene expression profiles in the presence and absence of WT1 by transient expression of *Cre* recombinase.

karotyptically normal, was chosen for the second round of gene targeting. The linearised targeting vector, *pWT1LoxP24*, was electroporated into the WT1 heterozygote null ES clone. Cells were selected with G418 for 7 days. 101 clones were isolated which survived G418 selection and screened via Southern blot, using a *BglII* digestion of genomic DNA and hybridisation with a 5' flanking probe (Figure 5.2 (a)). From the analysis, all the clones exhibited 6 kb and 2 kb fragments, indicating that none of the clones were correctly targeted with *pWT1LoxP24* (Data not shown).

The experiment was repeated again and 227 clones were isolated. By increasing the number of clones picked from the transfection, it was hoped that the probability of isolating a correctly targeted clone would be increased. The clones were analysed using the same genomic digestion and Southern blot hybridisation. From the 227 clones analysed, one clone, 14.17.74, was identified as being homologously targeted, by the presence of the expected 4 kb and 2 kb fragments (Figure 5.2(b)).

The next step was to transiently transfect the targeted clone, 14.17.74, with *pMC-Cre*. As demonstrated from targeting of the first allele and the generation of floxed clones described in Chapter 3, the transient expression of *pMC-Cre* and counter selection in gancyclovir (GANC), results in either type I or type II recombination. If the outcome is type I recombination, (recombination between the *loxP* sites flanking the selection cassette) then a conditionally targeted ES cell clone will be generated with exon 1 of *WT1* flanked by *loxP* sites. This clone could then be used in subsequent studies, where the loss of WT1 gene expression would be achieved through the re-expression of *Cre* recombinase (Figure 5.1). From type II recombination (recombination between the two outer *loxP* sites) a WT1 null ES clone would be generated, due to the excision of exon 1 and removal of the WT1 transcriptional and translational start sites (Figure 5.2).

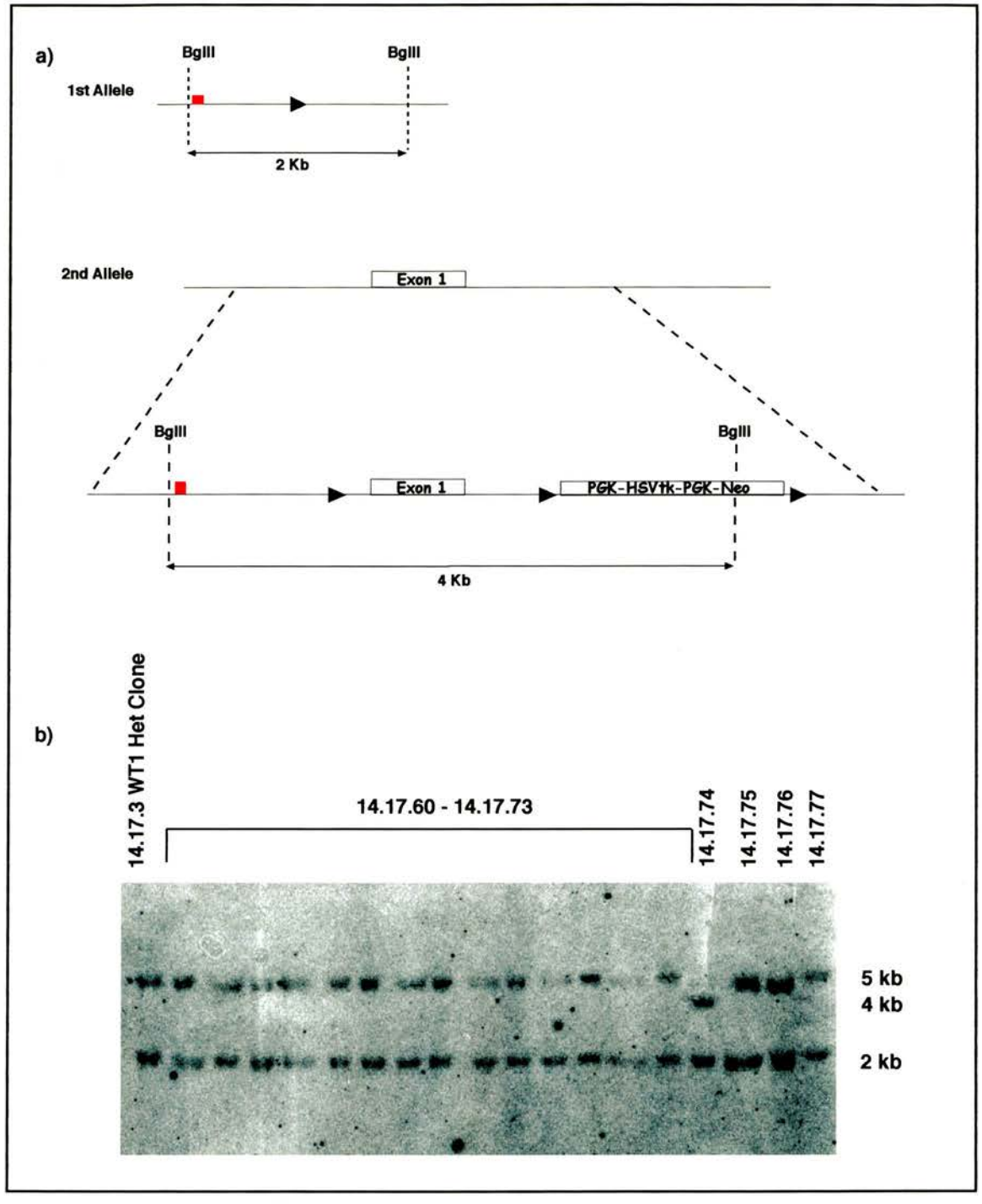


Figure 5.2 Targeting of the Second Wild-type *WT1* Allele with *pWT1LoxP 24* Targeting Construct

a) Figure showing the targeting of the second wild-type *WT1* allele with the *pWT1LoxP24*

b) Southern blot analysis confirms 14.17.74 as being homologously targeted with *pWT1LoxP24*. 10 μg of genomic DNA was digested with *BglII* and a Southern blot of the digested DNA was carried out using a 5' probe (red box). The first allele deleted using the *Cre/LoxP* system generates a 2 kb band, whilst correct targeting of the remaining wild-type allele with the *pWT1LoxP24* construct generates a 4 kb band.

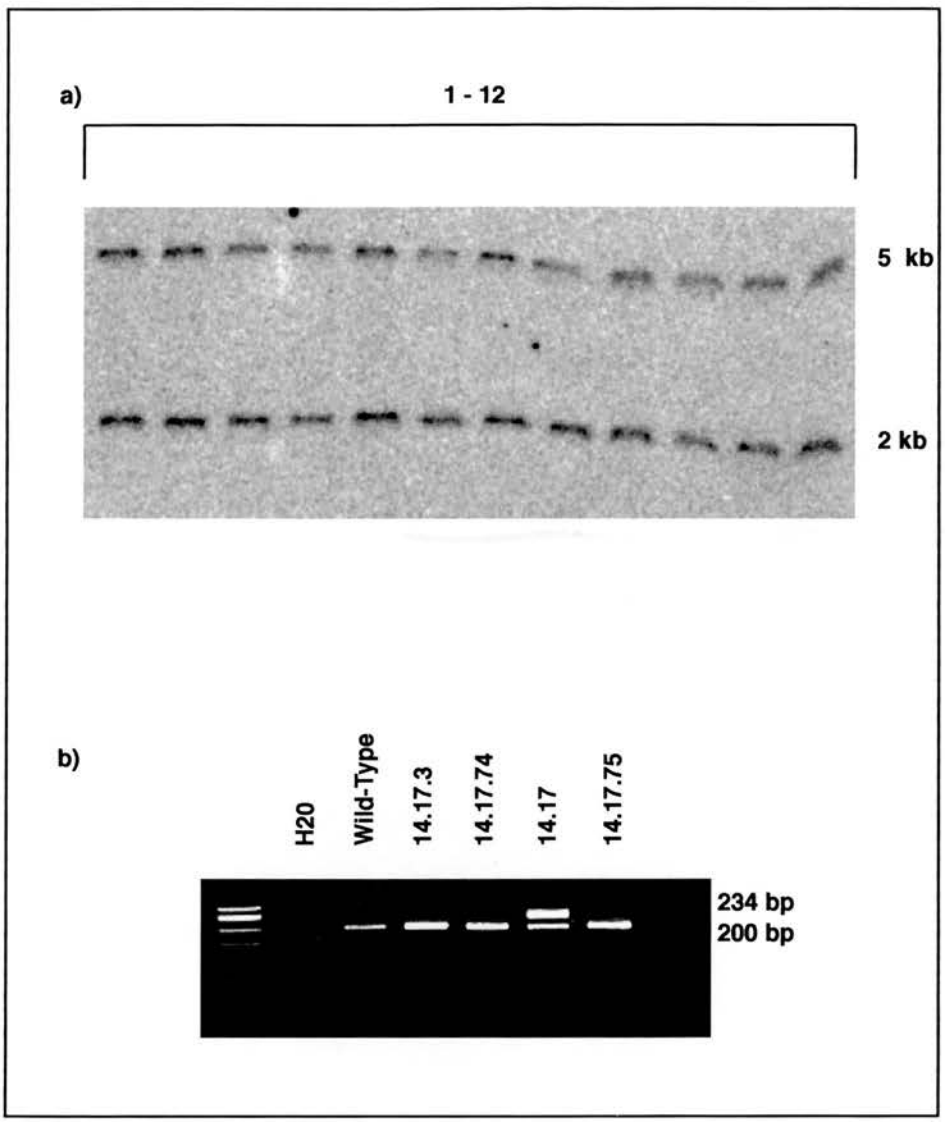


Figure 5.3 Transient Expression of *Cre* Does Not Result in Type II Recombination of the Homologously Targeted ES Cell Clone, 14.17.74.

a) Representative Southern blot of clones selected following transient expression of *Cre*. Gancyclovir resistant clones were picked following 10 days of selection. 10 μg of genomic DNA from the clones was digested with *BglII* and a Southern blot of the digested DNA was carried out using the 5' probe. All of the subclones exhibited only type I recombination (removal of the selectable marker cassette).

b) PCR analysis across the position where the 5' *LoxP* site resides demonstrates that the clone 14.17.74 does not have a *LoxP* site at this position.

Following the transient expression of *pMC-Cre*, 40 GANC resistant clones were analysed by Southern blot using the same *BglII* genomic digest of DNA and 5' flanking probe. All of the clones analysed displayed 6 kb and 2 kb fragments, indicating that the *Pgk-HSVtk-Pgk-Neo* selection cassette had been successfully removed (Figure 5.3 (a)). However, none of the clones analysed by Southern blot analysis had undergone type II recombination. This event would have generated a single band of 2 kb by Southern blot using the genomic digest and probe described above. Given that 80% of the clones in the targeting of the first allele and *Cre* treatment under went full deletion of the targeting vector, this was a surprising result.

Due to the absence of type II recombination, the next step was to analyse the parental clone 14.17.74, using primers spanning the 5' *loxP* site to ensure its presence in the clone. PCR analysis determined that 14.17.74 did not contain the 5' *loxP* site (Figure 5.3 (b)). This suggested that the recombination complex formed during homologous recombination had resolved downstream of the 5' *loxP* site, thereby not incorporating this site.

5.2.2 Targeting Using *pKreWT1* Construct

Due to the difficulty associated with targeting the second wild-type allele with the *pWT1LoxP24* targeting vector, the strategy chosen to generate WT1 null ES cells was changed. Instead of employing the *Cre/LoxP* system, the approach was to now generate WT1 null ES cell lines using the original targeting vector employed in the production of the WT1 mouse knockout (gift from Dr J Kreidberg; Kreidberg *et al.*, 1993). Correct homologous targeting, would in one step, generate a WT1 null ES cell line. Targeting of the remaining *WT1* locus with this replacement-type targeting vector, *pKreWT1*, deletes the first exon and part of the 5' upstream sequence, and replaces this with a neomycin cassette (Figure 5.4). The targeting vector contains a *HSVtk* cassette at the 3' end of the

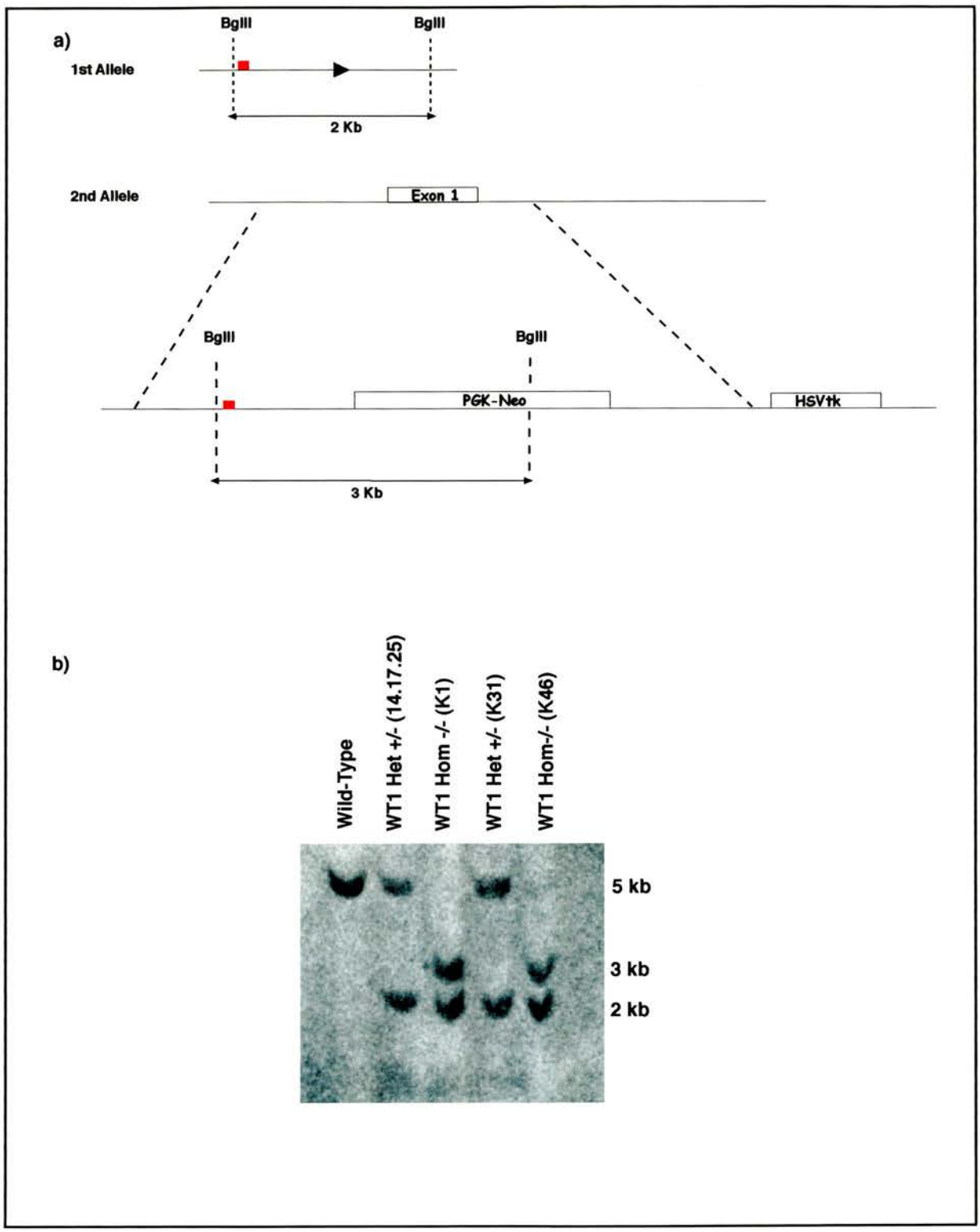


Figure 5.4 Generation of WT1 Homozygous Null ES Cells using the *pKreWT1* Targeting Construct

a) Figure showing the generation of WT1 null ES cells, by targeting the second wild-type allele with the *pKreWT1* construct

b) Southern blot analysis confirms K1 and K46 as being correctly targeted with the targeting vector. 10 μg of genomic DNA was digested with *BglII* and a Southern blot of the digested DNA was carried out using a 5' probe (red box). The first allele deleted using the *Cre/LoxP* system generates a 2 kb band, whilst correct targeting of the remaining wild-type with the *pKreWT1* construct generates a 3 kb band by Southern blot analysis.

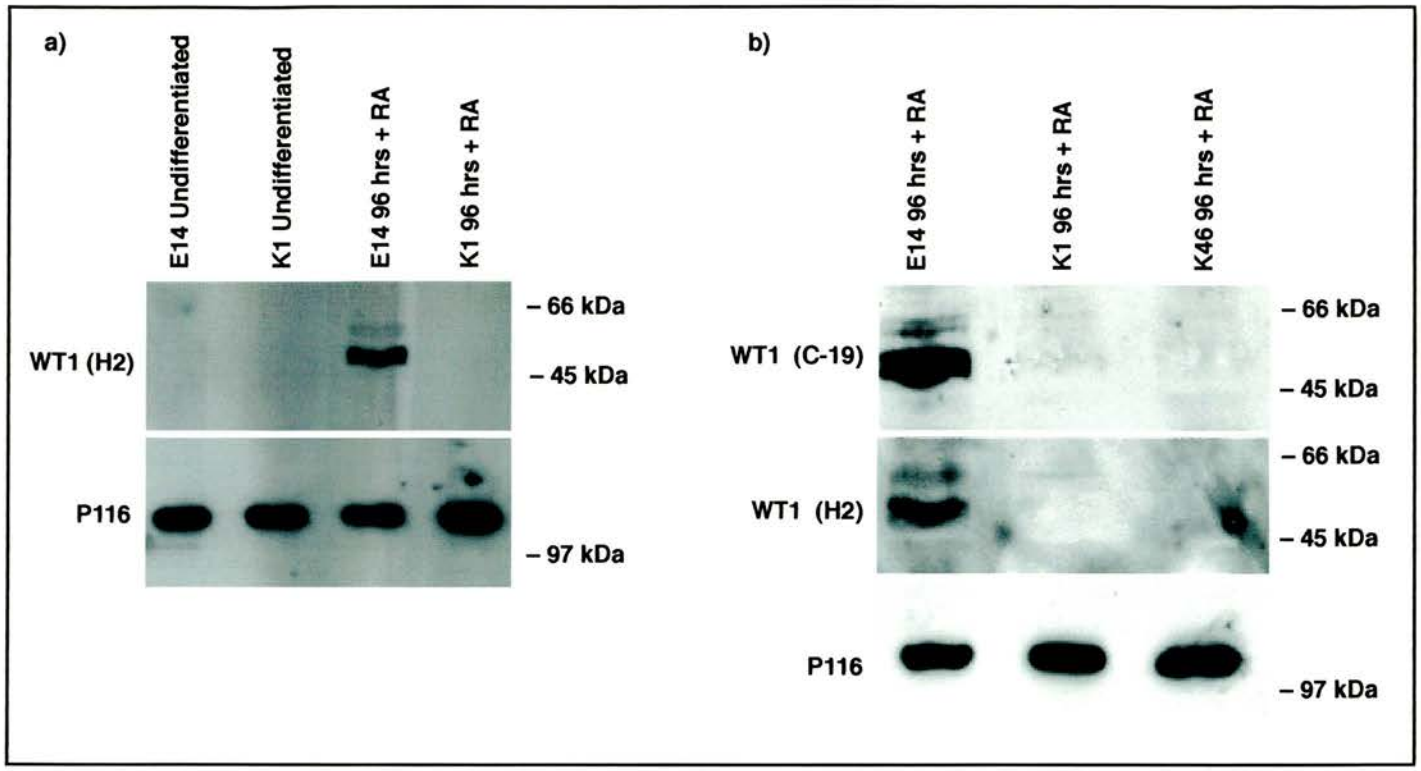
vector, which is used for positive-negative selection to reduce the background effects caused by random insertion of the construct into the genome.

Following the transient transfection of the parental clone, 14.17, with *pMC-Cre*, clones were selected in which type II recombination had occurred. Using one of these clones, 14.17.25, linearised *pKreWT1* targeting vector was introduced by electroporation and clones were grown in selection of both G418 and gancyclovir. Following 9 days of dual-selection, 123 clones were picked and analysed by Southern blot with the *BglII* genomic digest using the 5' probe. From the 123 clones tested, 2 were correctly targeted, as identified by the presence of a 3 kb and 2 kb bands expected for correct homologous recombination at the *WT1* locus (Clones K1 and K46) (Figure 5.4). Both clones were karotyped and shown to be normal (Data not shown).

5.2.3 Confirmation of WT1 Null ES Cells

As shown in Chapter 4, the treatment of wild-type ES cultures with *all-trans* retinoic acid (RA) induces high level expression of WT1. From the second round of gene targeting, two independent WT1 null ES clones were obtained (K1 and K46). To confirm that the sequential steps of *Cre-LoxP* inactivation of the first allele, followed by the insertion of a neomycin cassette in the second allele, did produce a WT1 null ES cell clone, K1 ($WT1^{-/-}$) cells were differentiated alongside E14 ($WT1^{+/+}$) ES cells with RA for 96 hours, and compared to K1 ($WT1^{-/-}$) and E14 ($WT1^{+/+}$) ES cells grown in the absence of RA. Following 96 hours, nuclear protein was harvested from the E14 and K1 RA treated clones, along with nuclear protein from the E14 and K1 clones in an undifferentiated state.

40 μg of nuclear protein from E14 and K1 in an undifferentiated state and 40 μg of protein from both clones differentiated for 96 hours with RA, were separated on a 10% SDS PAGE gel and transferred onto nitrocellulose. Antibodies for WT1, which are directed against the N-terminus (H2) and against the C-



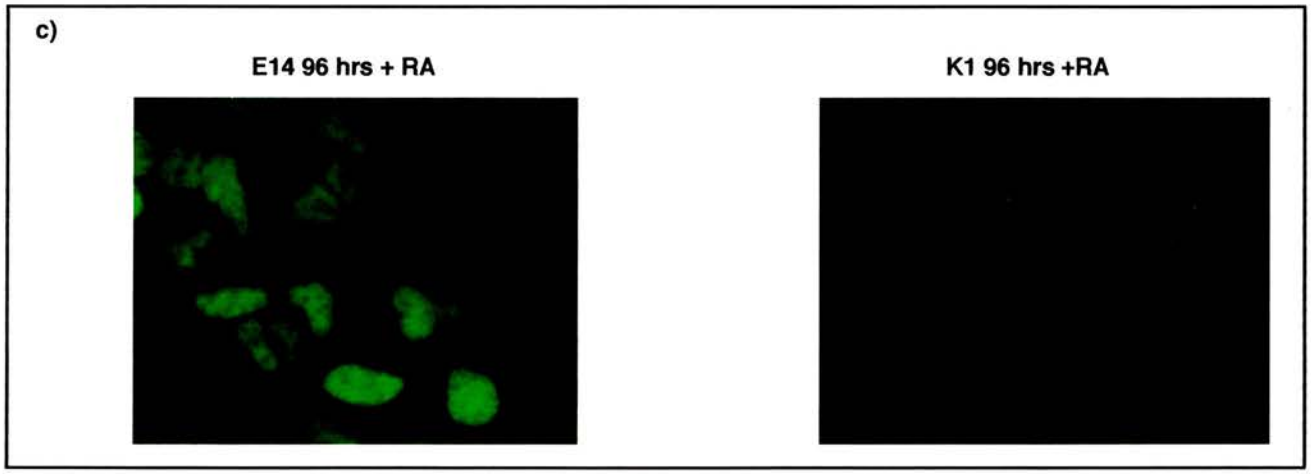


Figure 5.5 Western Blot Analysis and Immunofluorescence Confirms that K1 and K46 are WT1 Null ES Cell Lines

a) Nuclear protein was collected from E14 and K1 ES cell lines undifferentiated, and E14 and K1 ES cells treated with 1 μM RA for 96 hours. 40 μg of nuclear protein was separated on a 10% SDS PAGE gel and transfered to nitrocellulose. The blot was probed for WT1 using the N-terminal antibody (H2). To control for loading the blot was stripped and probed for p116, a U5 small nuclear RNP-associated protein.

b) Nuclear protein was collected from E14, K1 and K46 ES cells treated with 1 μM RA for 96 hours. 40 μg of nuclear protein was separated on a 10% SDS PAGE gel and transfered to nitrocellulose. The blots were probed for WT1 using both the N-terminal (H2) and C-terminal (C-19) antibodies. To control for loading the blot was stripped and probed for p116, a U5 small nuclear RNP-associated protein.

c) E14 and K1 ES cells were treated with 1 μM RA for 96 hours and immunofluorescence for WT1 was carried out using the C-19 antibody.

terminus (C-19) domains of WT1, were used to probe the blot for the presence of WT1.

In both E14 and K1 undifferentiated clones there is no detection of WT1 protein using either the C-19 or H2 antibody (Figure 5.5(a)). Following 96 hours of differentiation with RA, two doublets, representing WT1, can be detected in the E14 plus RA sample. These doublets arise from the alternative translation initiation sites and the presence/absence of the 17 amino insert arising from the alternative splicing of Exon 5. However in the lane containing protein from the WT1 null cells (K1) exposed to RA, there is no detection of WT1 protein after 96 hours of induction using both the C-19 and H2 antibodies (Figure 5.5 (a) and Figure 5.5 (b)). To ensure equal loading of the samples, the blots were reprobed with an antibody for P116. This shows that each of the lanes was loaded with approximately equal levels of nuclear protein. Therefore following induction by RA, K1 does not produce any detectable levels of WT1 protein as confirmed by Western blot (Figure 5.5).

To verify the Western blot result, and to observe whole cells rather than nuclear extract, E14 and K1 clones were grown on slides and either cultured in normal ES cell media, or differentiated for 96 hours in the absence of LIF and in the presence of RA. Cells were fixed, blocked and stained with both H2 and C-19 for immunofluoresence. In both undifferentiated clones there was no detectable expression of WT1 (Data not shown). Following 96 hours treatment with RA, approximately 70-80% of differentiated E14 ($WT1^{+/+}$) cells expressed relatively high levels of nuclear staining for WT1 (Figure 5.5 (c)). This is in contrast to the differentiated K1 ($WT1^{-/-}$) cell where there was no detection of WT1, by either C-19 or H2 antibodies (Figure 5.5 (c)). This confirms that K1 is an authentic WT1 null ES clone.

5.3 Morphology and Growth Rates

The suggested role for WT1 in cell proliferation and apoptosis prompted the examination of the growth rates of the WT1 null ES cells in comparison to the wild-type ES cells, in both the undifferentiated and RA differentiated states.

The growth rates of wild-type E14 ES cells ($WT1^{+/+}$) and WT1 null ES ($WT1^{-/-}$) cells (K1 and K46) were examined under culture conditions that maintain ES cells in an undifferentiated state. Equal numbers of cells were seeded at low densities in LIF containing medium, and cells were subsequently counted every 24 hours for a period of 96 hours. Counting of total cell numbers showed that, in comparison to E14 cells, the $WT1^{-/-}$ cells proliferated normally when grown in the presence of LIF (Data not shown). Morphologically, under these conditions $WT1^{-/-}$ ES cells were indistinguishable from wild-type ES cells (Figure 5.7). Also semi-quantitative RT-PCR analysis of Oct-4 expression, a key regulator of pluripotency in ES cells, showed that both E14 and K1 expressed comparable levels (Figure 5.16). Therefore in an undifferentiated state the loss of WT1 has no effect upon the normal morphology of embryonic stem cells.

In contrast to their normal behaviour in the undifferentiated state, $WT1^{-/-}$ ES cells exhibited differences in cell number following treatment with RA. The cell counts for E14 ES cells and WT1 null ES cells (K1 and K46) were monitored every 24 hours during the RA differentiation protocol (96 hours). The experiment was carried out in triplicate for each of the clones, and was also carried out on different passages of wild-type and $WT1^{-/-}$ cells (K1 and K46). The data is presented in figure 5.6.

At 24 hours and 48 hours the cell counts were similar for both E14 and the two $WT1^{-/-}$ ES cell clones. However by 72 hours there was an approximately 40% reduction in the cell numbers present in the $WT1^{-/-}$ clones (K1) in comparison to

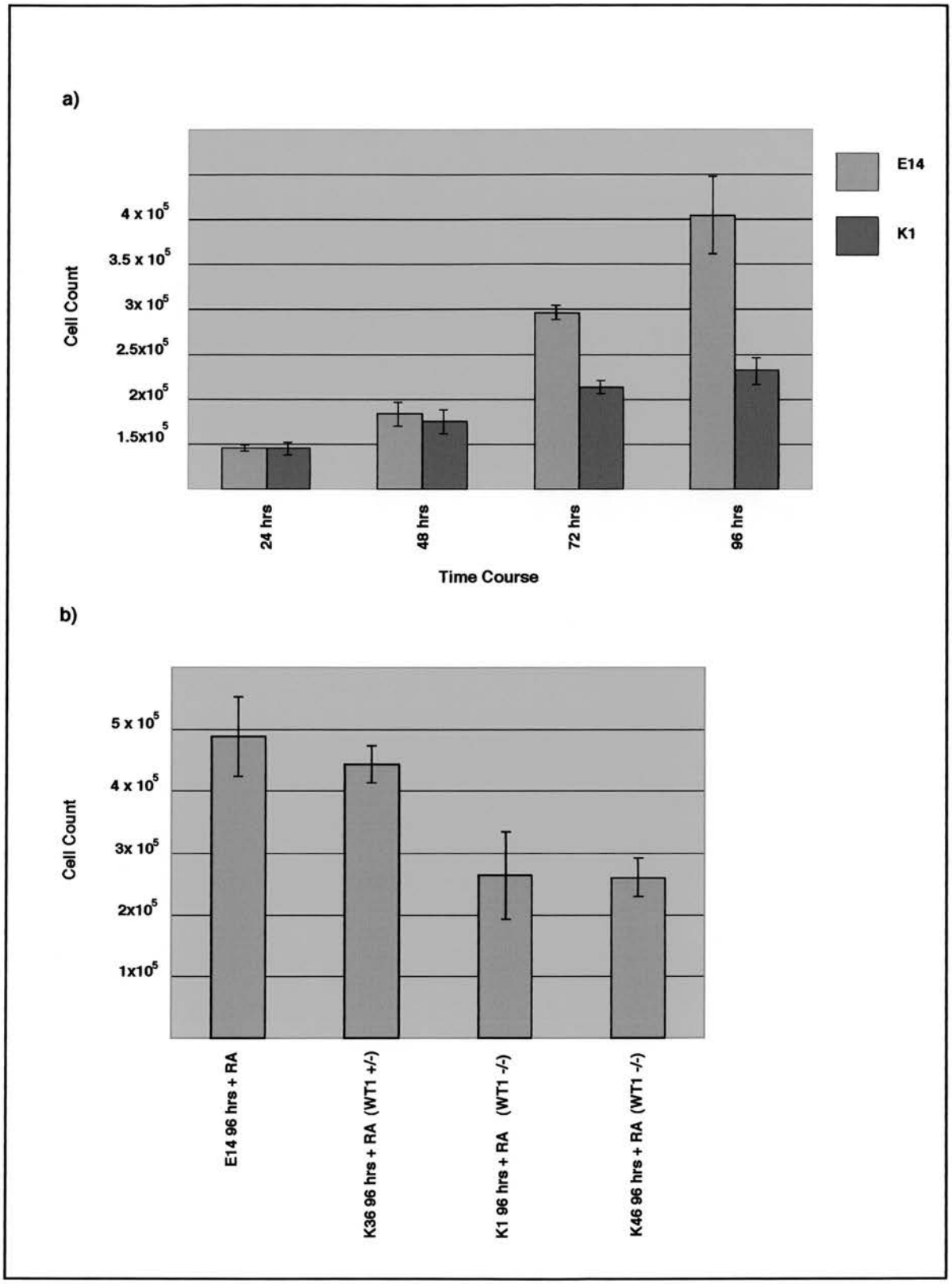


Figure 5.6 The Differentiation of WT1 Null ES Cells with RA Results in a Reduction of Cell Number Compared with Wild-type ES Cells

a) E14 and K1 (WT1-/-) ES cells were seeded in triplicate wells at a density of $1x10^4$. Both clones were differentiated in the absence of LIF and presence of 1 μM RA. At 24 hour intervals, the total cell numbers for each clone were counted.

b) E14, K36 (WT1+/-), K1 and K46 (WT1-/-) were seed in triplicate wells at a density of $1x10^4$. All clones were differentiated in the absence of LIF and presence of 1 μM RA. After 96 hours, the total cell numbers for each clone were counted.

Mean values from the triplicate experiments were plotted on the bar charts. Error bars correspond to +/- the Standard Deviation of the Mean.

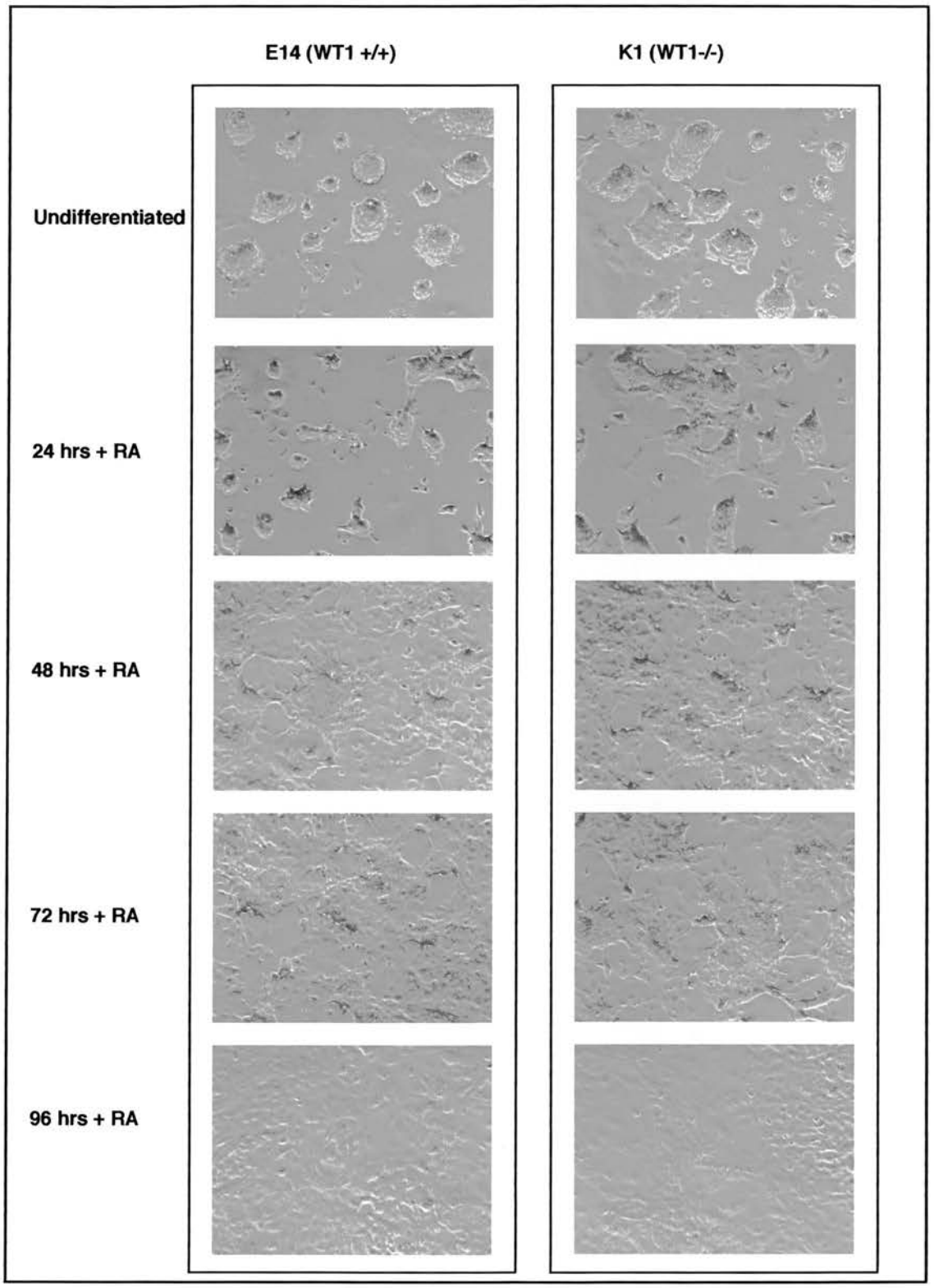


Figure 5.7 WT1 Null ES Cells have Similar Morphologies to E14 ES Cells in both the Undifferentiated State and During RA Differentiation

E14 and K1 ES cells were grown on coverslips and treated with RA for 96 hours. At 24 hour intervals, images were captured under phase contrast (Magnification x 10)

the wild-type ES cells. By 96 hours the difference between the RA treated $WT1^{-/-}$ ES cells and wild-type ES cells was around a 50 % reduction (Figure 5.7 (a)).

To ensure that the difference in cell number was not due to the effects of sequential rounds of gene targeting in the production of the $WT1^{-/-}$ ES cells, a WT1 heterozygote null ES cell clone ($WT1^{+/-}$) was also assayed in the RA differentiation protocol. The $WT1^{+/-}$ heterozygous null ES cell clone, K31, has been through the sequential steps of gene targeting and *Cre*-mediated recombination from the first round of gene targeting and has also been through the second round of electroporation and selection with the *pWT1Kre* plasmid. Therefore, this clone has been selected and recloned to the same extent as K1 and K46, but was not correctly targeted with the *pWT1Kre* targeting vector. Analysis of the levels of WT1 protein by Western blot demonstrated that the K31 cells expressed similar levels of protein as found in wild-type E14 ES cells (Data not shown). This is in agreement with the situation in WT1 heterozygote null mice in which WT1 is present at 95% of the level observed in wild-type mice (Guo *et al.*, 2002). Furthermore, the cell numbers for wild-type, $WT1^{+/-}$ and $WT1^{-/-}$ ES cells were counted following 96 hours of RA differentiation. The cell counts were similar for both the wild-type and $WT1^{+/-}$ clone, whilst there was a 50% reduction in cell numbers for the $WT1^{-/-}$ clones (K1 and K46) (Figure 5.6 (b)). Therefore the reduction in cell number in $WT1^{-/-}$ ES cells is likely to be a direct result of the absence of WT1.

The dramatic reduction in cell numbers observed during the differentiation of the WT1 null ES cells could reflect the loss of a particular lineage. However, the analysis of the morphologies of E14 and K1 ES cells every 24 hours during the differentiation protocol demonstrated that there was no obvious differences (in absence or presence of particular morphologies) represented in the two cultures across the time course (Figure 5.7).

5.4 Affymetrix Microarray Analysis

To analyse gene expression profiles of differentiating ES cells associated with WT1 loss-of-function, the Affymetrix Mouse Gene Chip U74A array was used. This array contains oligonucleotide probes specific for 6,000 known mouse genes present in the Mouse UniGene database, and 6,000 EST sequences. The expression profiles of the wild-type ES cells (E14) and one of the WT1 null ES cell lines (K1), was analysed at various time points in the RA differentiation protocol. The time points chosen for microarray analysis were (1) E14 and K1 ES cells in an undifferentiated state, (2) E14 and K1 ES cells differentiated for 48 hours with RA, and (3) E14 and K1 ES cells differentiated for 96 hours with RA.

In the undifferentiated state, there is no detectable expression of either WT1 RNA or protein in wild-type ES cells (Chapter 4, Figure 4.2). Therefore any differences in gene expression detected between E14 and K1 in the absence of RA exposure can be attributed to the effects of manipulation of the ES cells through gene targeting and subsequent passages *in vitro*, rather that an effect of the ablation of WT1. The next time point taken for analysis was RNA collected after 48 hours of differentiation with RA. This time point was chosen because in wild-type ES cells differentiated with RA, WT1 RNA is first detected after 48 hours. This sample would allow the analysis of genes that are effected immediately by the absence of WT1. Added to this is the analysis of the cell numbers after 48 hours of RA differentiation. At this time point, the cell counts between both E14 and K1 clones is similar. Therefore microarray analysis of this sample may indicate specific changes in gene expression, pointing towards any early cellular changes occurring in the K1 ES clone in comparison to the E14 ES clone. The final time point for analysis was RNA collected from the two ES clones following 96 hours treatment with RA. At this time point, 80% of wild-type ES cells are expressing high levels of WT1, and this represents the final point of the differentiation protocol.

For each time point, the experiment was carried out in triplicate. ES cells, E14 and K1, were seeded at equal concentrations and treated with RA for up to 96 hours. One RNA sample from each time point was sent for microarray analysis, whilst the duplicate samples were stored for verification of the findings from the arrays. Microarray hybridisations were carried out by Dr. Kevin Robertson at the Genomic Technology and Informatics (GTI), University of Edinburgh.

5.4.1 Normalisation of Arrays

The Affymetrix GeneChip arrays were scanned and analysed with Affymetrix MAS 5.0 software to identify transcripts that were classified as being absent, present or marginal. In addition to the unique probes present on the arrays, each array contains control probes (such as GAPDH) that control for the quality of the biotinylated RNA samples and hybridisation protocol. Based upon the hybridisation to the control probes, all of the 6 chips were of comparable quality (Data not shown). Next the expression data obtained from the 6 arrays was normalised to adjust the individual hybridisation intensities. In doing this, it permits meaningful comparisons to be made between the different RNA samples (Dr Kevin Robertson, personal communication).

5.4.2 Gene Expression Profiles of E14 and E14 Differentiated ES Cells

The differences in gene expression profiles between undifferentiated E14 ES cells and E14 cells treated with RA for 48 hours and 96 hours, were first analysed by scatter plot analysis. This graphical representation gives an idea of the global similarities or differences between the two RNA samples. In this representation, each point on the graph represents a single annotated gene or EST on the U74A array. The location of the point on the scatter plot is determined by the difference of signal intensity of this transcript from one RNA sample to another. Figure 5.8 shows the scatter plot for RNA collected from

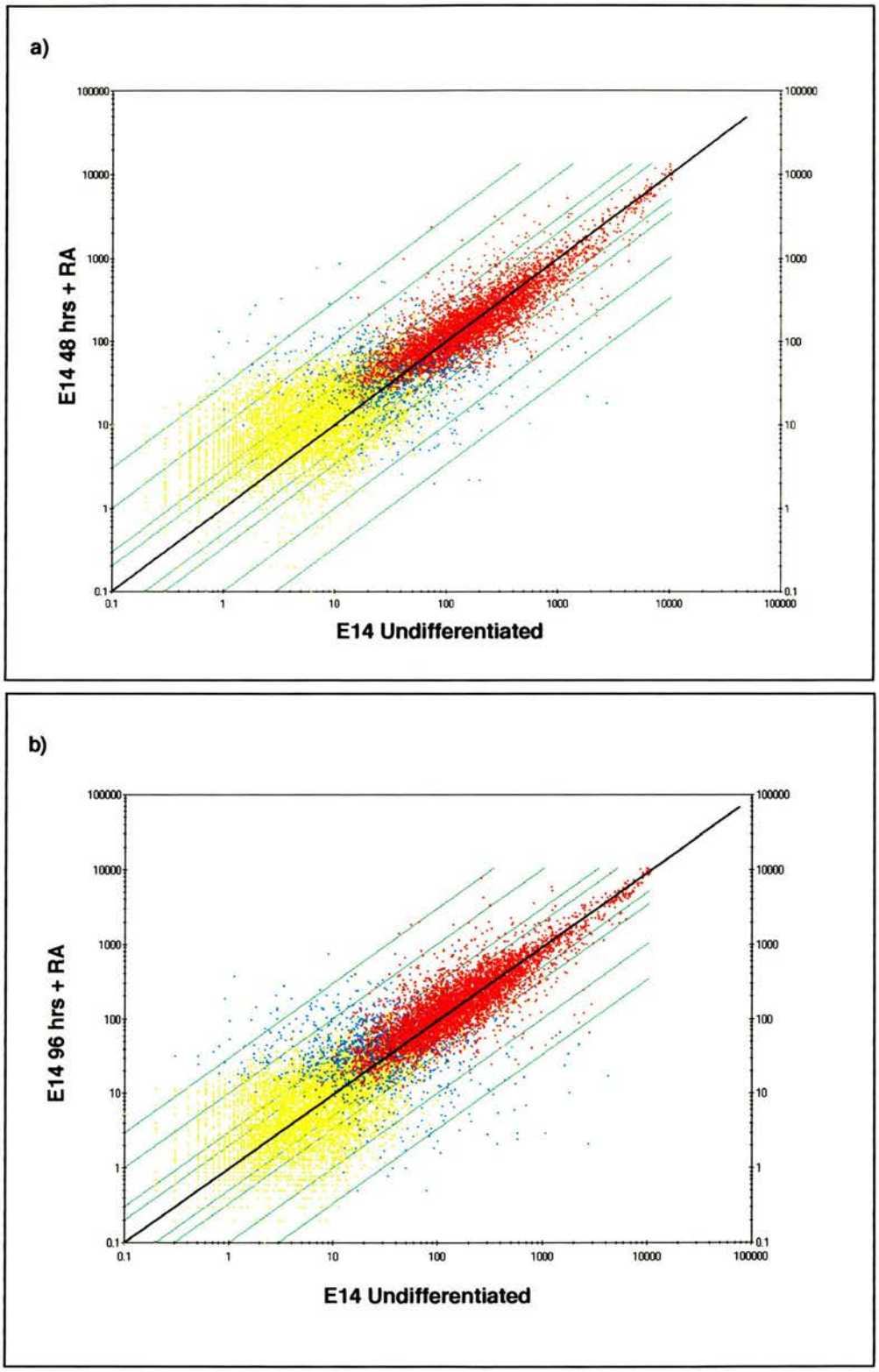


Figure 5.8 Scatter Plot Analysis of the Gene Expression Profiles of Wild-type E14 ES cells Treated with RA for 48 Hours and 96 Hours

a) Undifferentiated E14 ES cells compared to E14 ES cells differentiated for 48 hours with RA

b) Undifferentiated E14 ES cells compared to E14 ES cells differentiated for 96 hours with RA

Each point represents the normalised expression level of an individual transcript within both RNA samples. Yellow points denote transcripts that are not expressed in either RNA sample, blue represents transcripts that are absent in one of the two samples, whilst red points denote transcripts which are present in both samples.

undifferentiated E14 ES cells versus E14 ES cells treated with RA for 48 and 96 hours. The signal intensities of the array for RNA collected from undifferentiated E14 ES cells (X axis) is compared to the signal intensities obtain from the array for RNA collected from E14 ES cells treated for 48 hours with RA (Y axis). The distance that a point lies away from the midline (marked in black) represents the differential expression of a gene between two sample RNA populations. For example, a point that lies close to the midline represents a gene that is expressed at similar levels in the two RNA populations, whilst a point that lies further away from the line denotes a gene which is differentially expressed between the two RNA populations. The position of a point along the length of the line represents its level of expression in relation to other genes.

The points on the scatter plot are also colour coded. Red points represent transcripts that are classified as being present in both RNA samples. Blue points represent transcripts that the Affymetrix software has classified as being marginal, in that the software can not determine whether the transcript has hybridised to the array or not. Finally, yellow points denote transcripts that are not expressed in the two samples.

Comparison of the scatter plot analysis of the gene expression between E14 ES undifferentiated and E14 ES after 48 hours and E14 ES after 96 hours of RA treatment demonstrates that the distribution of points grows wider as ES cells differentiate under the influence of RA (Figure 5.8). This reflects the global change in gene expression as the homogenous ES cells differentiate to various lineages. To begin to analyse the data sets, an arbitrary value of fold changes of 2.5 or greater was chosen to represent genes that are differentially expressed between two samples. This value was used in all comparisons throughout the remainder of this chapter. Using this criterion, 842 genes were differentially expressed by 2.5-fold or greater between E14 ES cells and E14 ES cells differentiated with RA for 96 hours.

5.4.3 Genes Down-Regulated Following RA Differentiation of E14 ES Cells

Of the 842 genes differentially regulated in this system, 332 genes were down-regulated during the differentiation of E14 ES cells. Figure 5.9 represents the top 25 transcripts, ranked with regards to their fold change, that were detected as being present in the E14 ES cells in an undifferentiated state, and then absent in the E14 cells differentiated for 96 hours with RA. Therefore, the list represents genes whose expression is restricted to undifferentiated ES cells, and hence maybe associated with ES cell pluripotency. Among these transcripts are genes known to be crucial regulators of pluripotency in ES cells including Oct-4 (136-fold decrease), Rex-1 (34-fold decrease) and UTF-1 (66-fold decrease), a downstream target of Oct-4 (Nichols *et al.*, 1998; Ben-Shushan *et al.*, 1998; Okuda et al., 1998; Nishimoto *et al.*, 1999). The down-regulation of Oct-4 observed in the Affymetrix analysis confirms the RT-PCR data for Oct-4 as described in Chapter 4.

The transcript with the highest fold change was Embryonic stem cell specific 1 (Esg-1), a gene with no known sequence homologies or function. This gene was down-regulated by 209-fold in the E14 ES cells differentiated with RA for 96 hours, indicating that this gene maybe associated with ES pluripotency. Previously this gene was described as being down-regulated during retinoic acid differentiation of embryonic carcinoma (EC) cells, and also expressed uniquely in ES cells and pre-implantation embryos (Astigiano *et al.*, 1991; Bierbaum *et al.*, 1994). The recent work using Affymetrix Genechip arrays on a variety of stem cells populations identified genes that were enriched in embryonic stem cells (Santos *et al.*, 2002). When the genes were ranked in accordance with their enrichment, the top ranking transcript, even more abundant than Oct-4, was Embryonic stem cell specific 1 (Esg-1) (Santos *et al.*, 2002). Recent work on the gene expression profile of murine ES cells, extraembryonic-restricted trophoblast stem cells and terminally differentiated embryonic fibroblast cells, have also identified Esg-1 as being associated with ES pluripotency

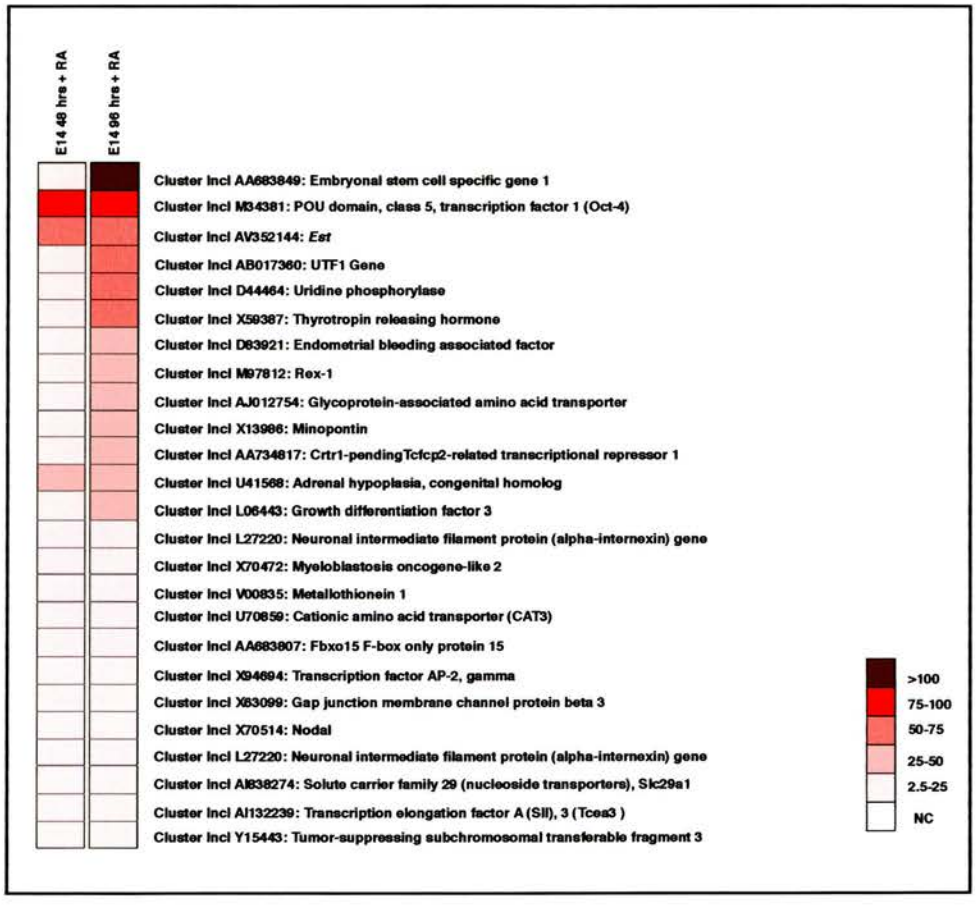


Figure 5.9 Top 25 Transcripts Down-Regulated During RA Differentiation of E14 ES cells

The top 25 down-regulated transcripts, which were present in undifferentiated E14 ES cells and absent in RA treated ES cells following 96 hours, were ranked according to their fold-change. Included is their colour-coded fold-change decrease following 48 hours of treatment with RA.

(Tanaka *et al.*, 2002). Data presented by the authors indicated that Esg-1 may lie downstream of the LIF-Stat3 and Oct3/4 pathways (Tanaka *et al.*, 2002).

As discussed above, Oct-4, Rex-1 and UTF-1 represent genes which are known to be crucial regulators of ES cells pluripotency, whilst Esg-1 represents a novel gene that may also be involved in this process. In the top 25 down-regulated genes, there is also an EST (AV352144) which is down-regulated 90-fold during the differentiation of ES cells. A BLAST search against the murine database indicates that this EST has no know annotated gene, however it does contain a region of homology with human Nkx2.5 domain. This transcript may belong to the same family of proteins as EHOX, a recently isolated novel paired like homeobox containing gene (Jackson *et al.*, 2002). The EST has been detected in libraries derived from ES cells, testis, morula, blastocyst and gonad, and therefore represents a novel EST that is enriched in stem cell lineages. Present in the list of transcripts are genes that have not been previously associated with ES cells pluripotency (Figure 5.9).

5.4.4 Genes Up-Regulated Following RA Differentiation of E14 ES Cells

Of the 842 genes that were differentially expressed in this system, 510 were up-regulated during the differentiation of E14 ES cells. The top 30 transcripts, ranked in order of their fold change, are shown in Figure 5.10. Some of these transcripts represent direct targets of the retinoic acid pathway, or are involved in the regulation/modulation of retinoic acid signalling, including plasminogen activator (19-fold increase) (Chen and Gudas, 1996) and cytochrome P450 26 retinoic acid A1 (Cyp26A1) (10-fold increase) (Freemantle *et al.*, 2002). Present in the list of the 30 induced transcripts are several markers of neuronal lineages. Sox11, *in vivo*, is expressed predominantly in the neural epithelium of the central nervous system (Uwanogho *et al.*, 1995; Hargrave *et al*, 1997). Ephrin B1 is expressed in the developing brain, where it has been

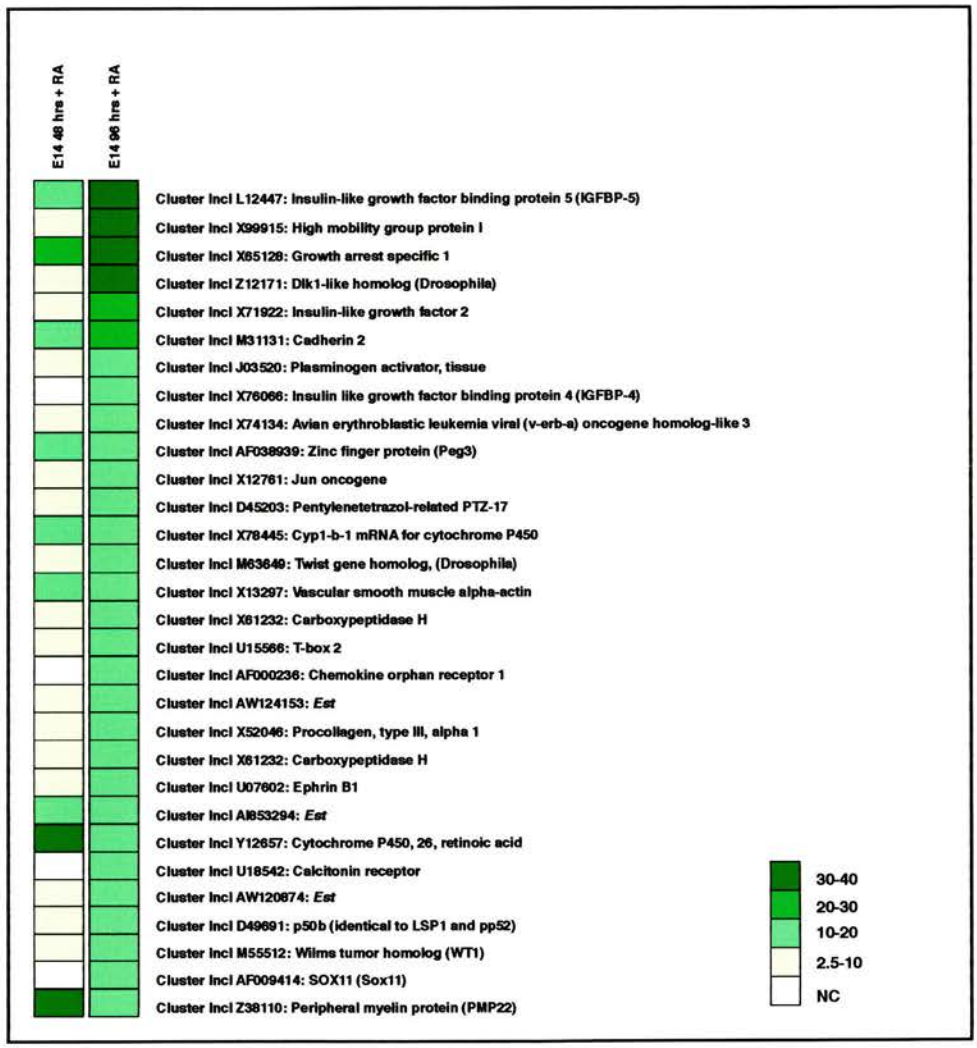


Figure 5.10 The Top 30 Transcripts Up-Regulated During RA Differentiation of E14 ES cells

The top 30 transcripts which were up-regulated during the RA differentiation of E14 ES cells were ranked in order of there fold change at 96 hours. Included is their colour coded fold-change increase following 48 hours of treatment with RA.

implicated in axonal pathfinding during the formation of neuronal networks (For review see Mellitzer *et al.*, 2000; Moreno-Flores *et al*, 2002), whilst Peripheral Myelin Protein (PMP22) encodes a protein component of the myelin sheath of peripheral nerves (Jetten and Suter, 2000). The up-regulation of neuronal markers seen here is consistent with the findings from the RT-PCR analysis in Chapter 4, which showed an increase in expression of additional neuronal markers, namely nestin and Pax6.

Additionally, in the list of transcripts induced by RA are transcripts which show a mesodermal/mesenchymal expression *in vivo*. This includes the *Drosophila* Twist gene homologue (13-fold increase) which is expressed in head mesenchyme, somites and limb buds, and is required for the development of these structures (Wolf *et al.*, 1991; Chen and Behringer, 1995) and vascular smooth muscle alpha-actin (13-fold increase). There are also transcripts which have a diverse expression pattern *in vivo* including Dlk1 (30-fold increase), an inhibitor of adipogenesis that also regulates embryonic pancreas formation and differentiation of beta-cells (Carlsson *et al.*, 1997) and T-Box2 (Tbx2) (12-fold increase) which is expressed primarily in kidney, lung, and placenta (Campbell *et al.*, 1995).

Several genes encoding for transcription factors or DNA binding factors are up-regulated in the E14 differentiated cells. The expression of the AP-1 component, c-Jun, is increased 14-fold during the differentiation of ES cells. This induction is in agreement with the reports of c-Jun induction in RA differentiation of EC cells and ES cells (Pankov *et al.*, 1994; Kelly and Rizzino, 2000). c-Jun has been shown to activate a number of lineage specific genes, including vimentin. An up-regulation of vimentin was seen in the Affymetrix arrays during the differentiation process, and antibody staining in Chapter 4 demonstrated that the majority of vimentin positive differentiated ES cells co-expressed WT1. Another gene up-regulated was the high mobility group protein 1 (*HMG-1*) (34-fold). HMG-1 belongs to a family of highly conserved chromatin-associated

nucleoproteins that bend DNA and facilitate the binding of various transcription factors to their cognate DNA sequences (Jayaraman *et al.*, 1998).

By 96 hours there is a 10-fold induction in the levels of WT1. Across the time points taken, there is a gradual increase in the levels of WT1 expression, from undetectable expression in the undifferentiated state, to a 4-fold induction at 48 hours and finally a 10-fold induction by 96 hours. This induction pattern of WT1 observed in the Affymetrix array data is in agreement with the RT-PCR data presented in Chapter 4 showing the expression profile of WT1 RNA.

In summary, the up-regulation of genes from multiple lineages following RA mediated differentiation of ES cells suggests that the effects of RA are quite heterogeneous (Figure 5.10). Whilst previous reports have suggested that RA directs ES cells towards a predominantly ectodermal fate (Bain *et al.*, 1995, For review see Guan *et al.*, 2001), this more extensive preliminary study using Affymetrix Genechip microarrays suggests that other lineages, such as mesoderm may be present in the monolayer cultures. However the strong up-regulation of WT1 following RA induction makes this system ideal to investigate the effects of WT1 loss-of-function on gene expression, and to identify candidate WT1 regulated genes.

5.5 Comparison of Gene Expression Profiles between K1 and E14 ES Cells

5.5.1 Undifferentiated E14 and K1 ES Clones

In comparing the gene expression profiles of the two clones from the three time points (0, 48, 96 hours) the data from the Affymetrix Genechip arrays were first analysed using scatter plot analysis. In the undifferentiated state, the scatter plot analysis reveals a very tight distribution pattern along the midline. This indicates that the expression profile of the majority of genes is unchanged

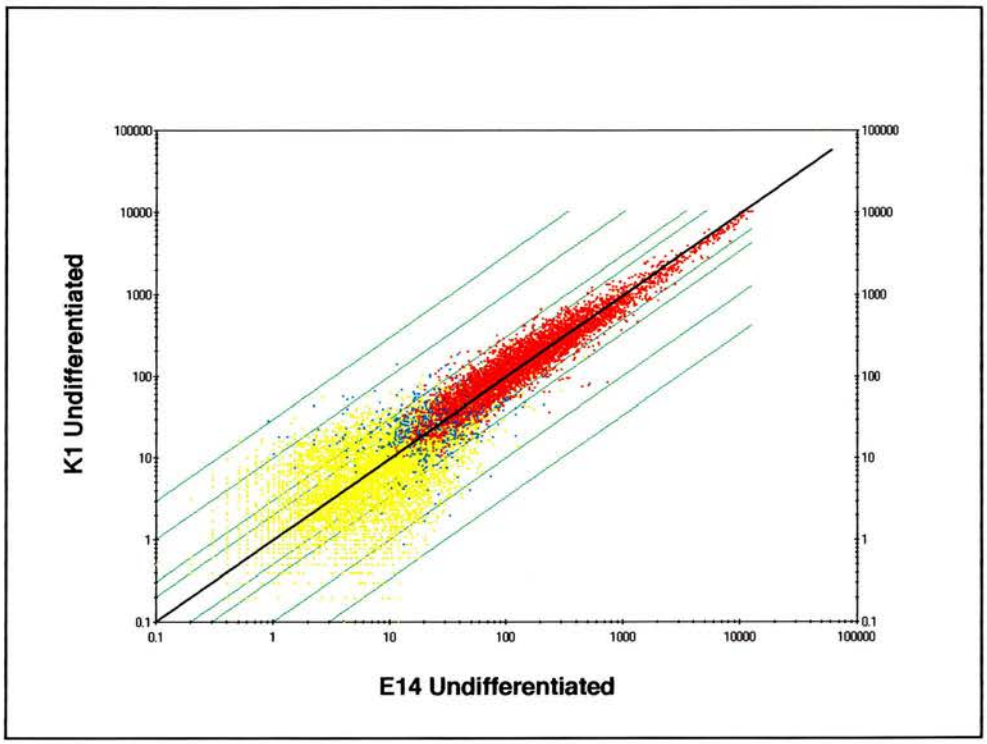


Figure 5.11 Scatter Plot Analysis of the Gene Expression Profiles of E14 and K1 ES Cells in an Undifferentiated State Indicates that the Samples have Similar Gene Expression Profiles

Each point represents the normalised expression level of an individual transcript within both RNA samples. Yellow points denote transcripts that are not expressed in either sample, blue represents transcripts that are absent in one of the two samples, whilst red points denote transcripts which are present in both samples.

between E14 and K1 in the undifferentiated state (Figure 5.11). Although there were some differences between E14 and K1 ES cells in the undifferentiated state, there was no difference in the levels of expression of the key genes associated with ES cell pluripotency e.g. Oct-4, UTF-1, Rex-1. This indicates that the absence of WT1 has no affect upon the normal physiology of undifferentiated ES cells. This agrees with the findings described in section 5.3, in that the gross analysis of the WT1 null ES cells demonstrates that they have similar morphology and growth rates.

5.5.2 E14 and K1 ES Cell Clones at 48 hours of Differentiation

As with the comparison of gene expression profiles of the clones in an undifferentiated state, the scatter plot analysis after 48 hours of treatment with RA also reveals a tight distribution pattern of transcripts along the midline (Figure 5.12). This indicates the gene expression profiles at 48 hours is again highly similar between the two clones. At 48 hours there were 61 genes which were differentially expressed 2.5-fold or more between E14 and K1 (Figure 5.13). Green represents the transcripts that are up-regulated in the K1 clone in comparison to the E14 clone, whilst red depicts transcripts which are down-regulated in the K1 clone in comparison to the E14 clone. White denotes transcripts that have not changed between the two clones. Of the 61 transcripts, 24 were down-regulated, and 34 transcripts up-regulated in the K1 clone in comparison to the E14 clone. From this list of 61 transcripts, 27 represent ESTs with little or no known homologies.

5.5.3 E14 and K1 ES Cell Clones at 96 hours of Differentiation

Scatter plot analysis of the RNA samples collected after 96 hours of treatment with RA indicated that the gene expression profiles between the two clones was again highly similar (Figure 5.14). This was also supported by the fact that only 41 genes were differentially expressed 2.5-fold or more between the two samples (Figure 5.15). Of the 41 transcripts, 17 genes were up-regulated and 24

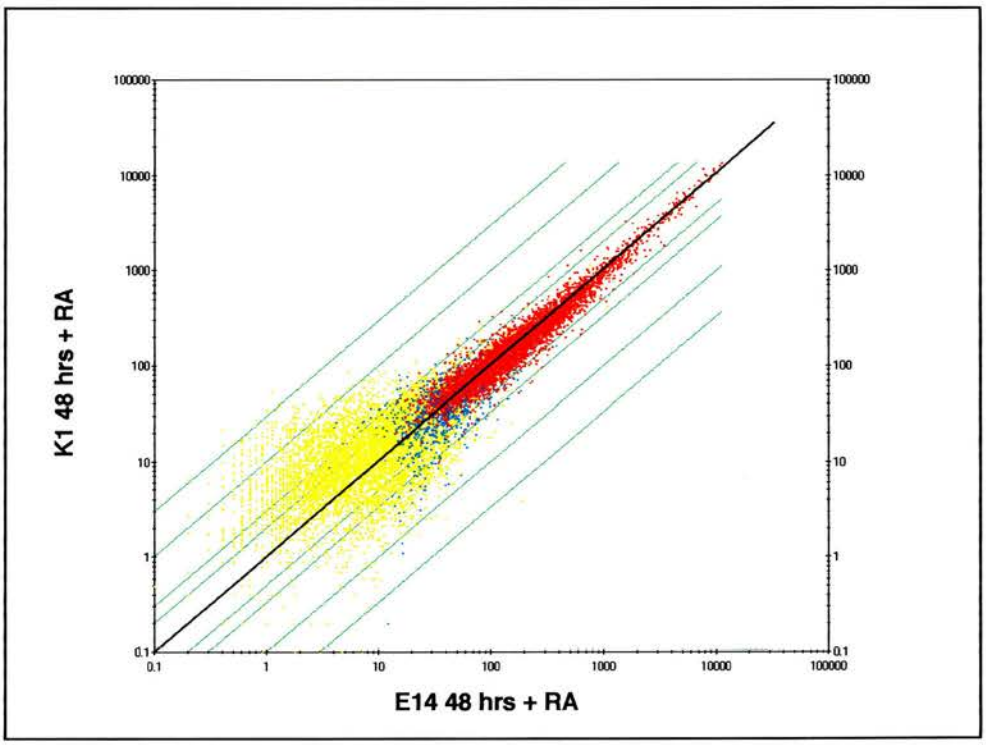


Figure 5.12 Scatter Plot Analysis of the Gene Expression Profiles of E14 and K1 ES Cells Treated with RA for 48 Hours Indicates that Both Clones have Similar Gene Expression Profiles

Each point represents the normalised expression level of an individual transcript within both RNA samples. Yellow points denote transcripts that are not expressed in either sample, blue represents transcripts that are absent in one of the two samples, whilst red points denote transcripts which are present in both samples.

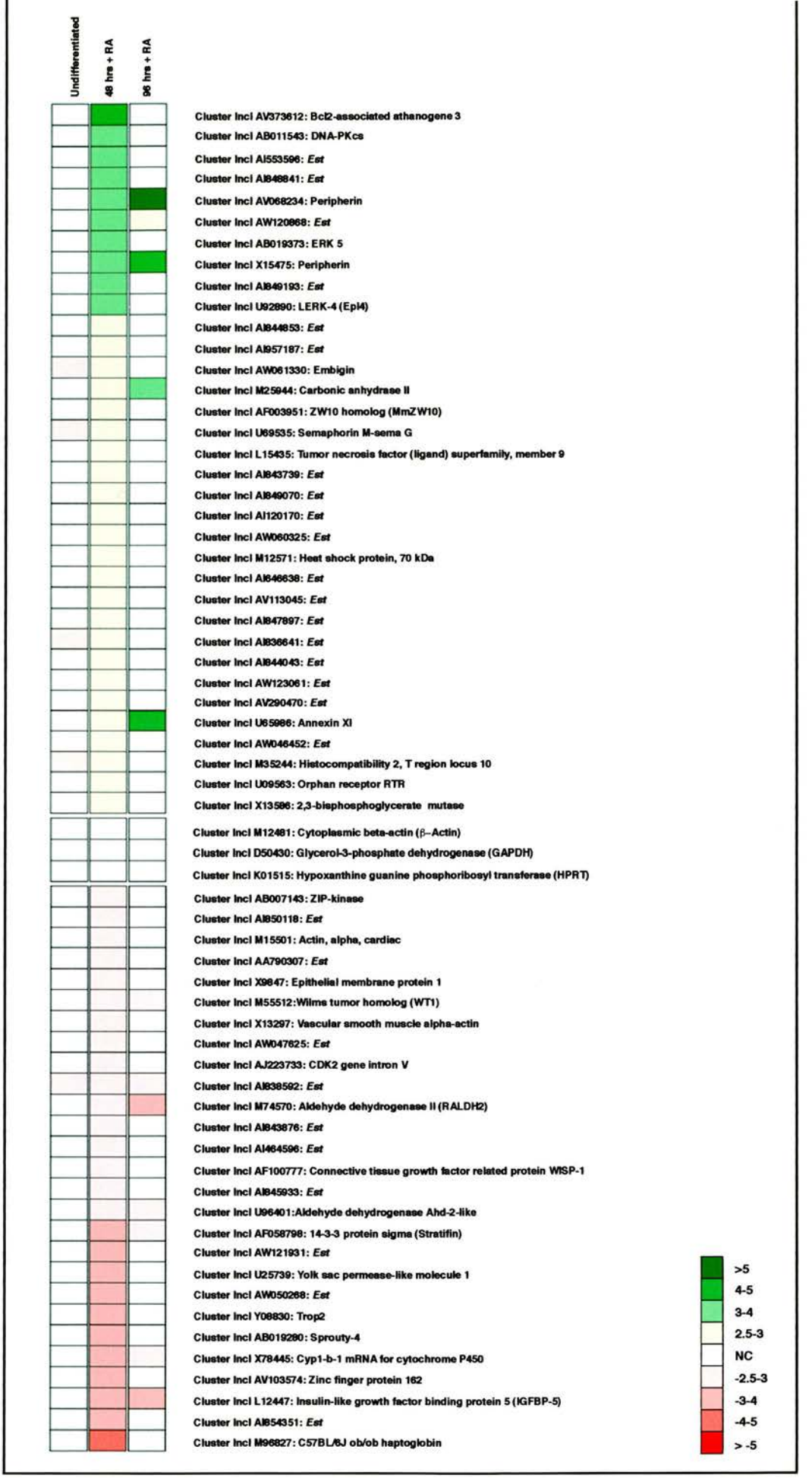


Figure 5.13 Transcripts Differentially Expressed at 48 hrs in the K1 (WT1-/-) ES clone

Transcripts which were up-regulated (green) or down-regulated (red) by 2.5 fold or greater in the K1 ES clone following 48 hours of RA treatment, were ranked according to their fold-changes. Their fold-change at 96 hours is also shown.

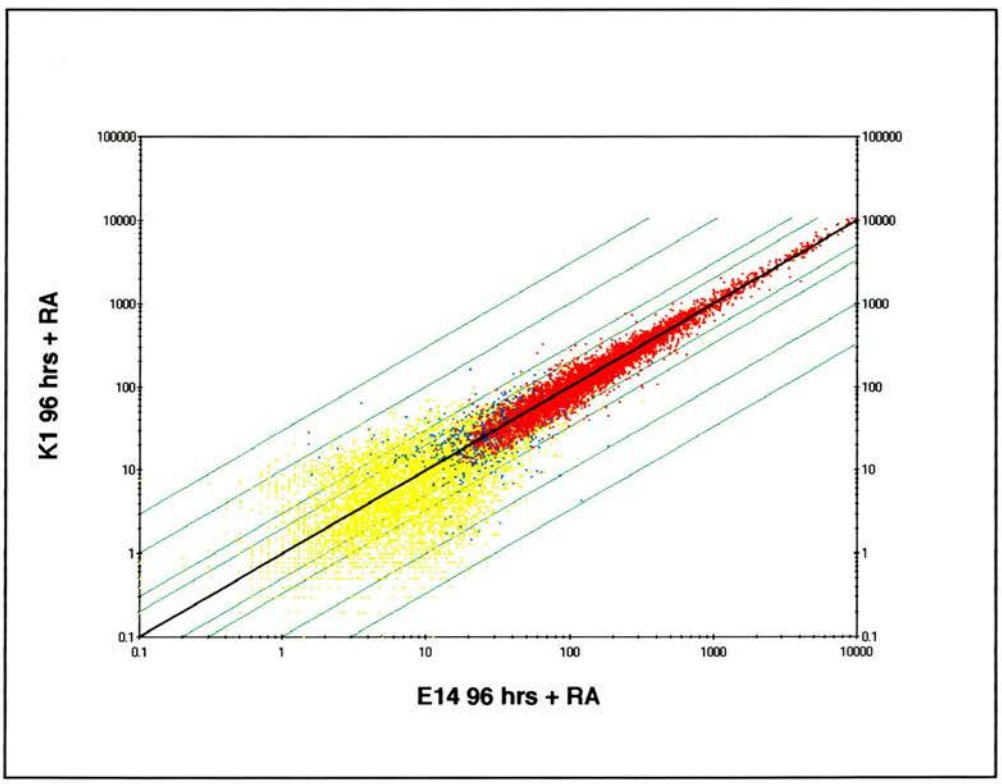


Figure 5.14 Scatter Plot Analysis of the Gene Expression Profiles of E14 and K1 ES Cells Treated with RA for 96 hours, Indicates that Both Clones have Similar Gene Expression Profiles.

Each point represents the normalised expression level of an individual transcript with both RNA samples. Yellow points denote transcripts that are not expressed in either sample, blue represents transcripts that are absent in one of the two samples, whilst red points denote transcripts which are present in both samples.

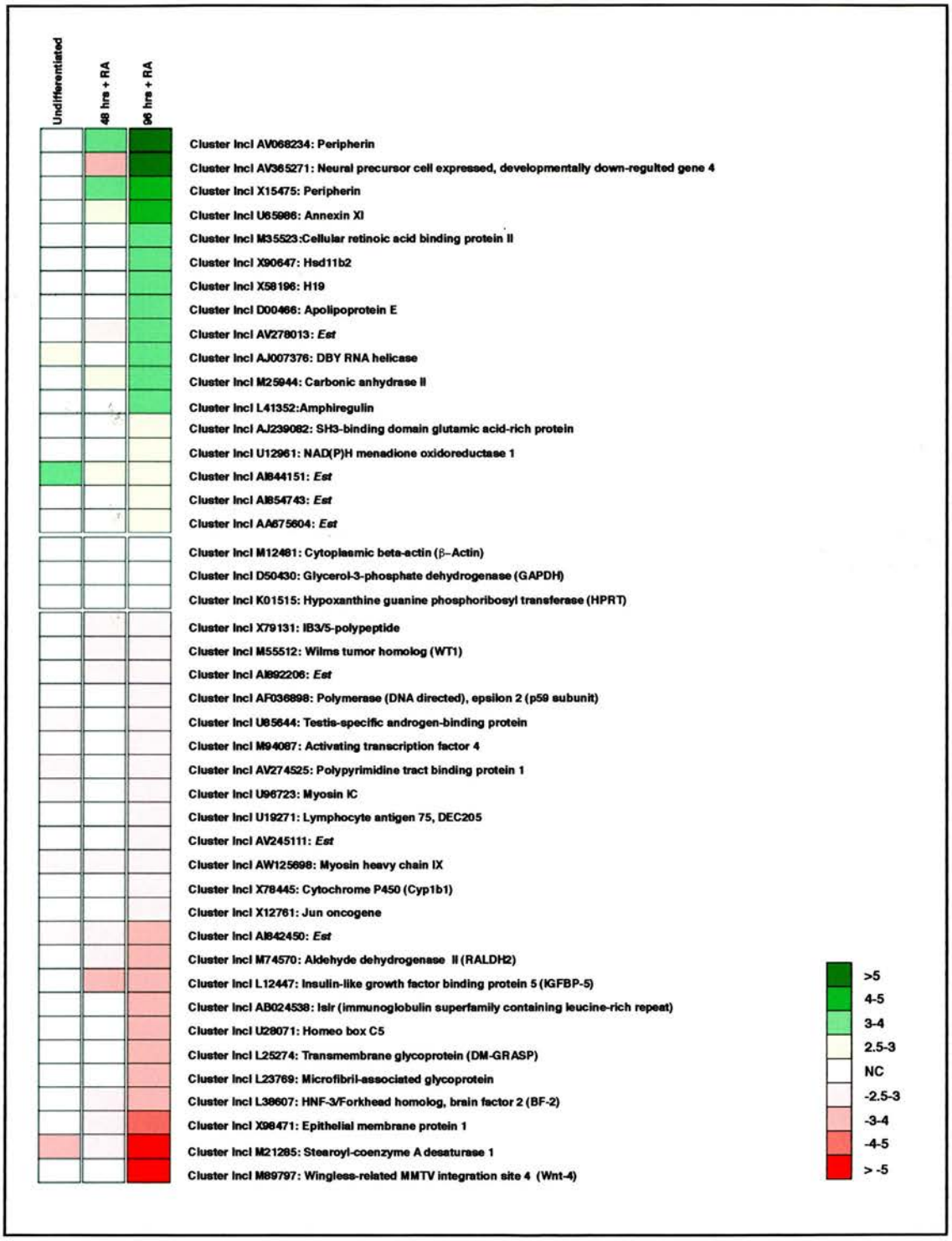


Figure 5.15 Transcripts Differentially Expressed at 96 hours in the K1 (WT1-/-) ES clone

Transcripts which were up-regulated (green) or down-regulated (red) by 2.5 fold or greater in the K1 ES clone following 96 hours of RA treatment, were ranked according to their fold-change.Their fold-change at 48 hours is also shown.

were down-regulated in the K1 sample compared to the E14 sample. The majority of the transcripts are annotated genes, with only 7 representing unknown ESTs.

5.5.4 Differentially Expressed Transcripts in the WT1 Null ES Cells

Overall there were 102 transcripts differentially expressed between the WT1 null ES cells and the wild-type ES cells at the two time points analysed. From this list, it is apparent that there are obvious alterations in the expression profiles of specific gene families. Also there are appears to be equal numbers of genes differentially up-regulated or down-regulated across the two time points in the WT1 null ES cells, indicating that WT1 could be acting as a transcriptional activator as well as a transcriptional repressor in the regulation of these candidate target transcripts. Some of the transcripts from the list are discussed in context with a possible physiological relevance to WT1.

5.5.4.1 Cellular Stress

In considering the transcripts that are differentially expressed, it would appear that the K1 clone maybe exhibiting a response to some form of cellular stress. Although both clones exhibit similar cell counts at 48 hours, rapid cellular changes must be occurring over the next 24 hours, as by 72 hours there is a 50% reduction in the cell numbers in the K1 clone. The up-regulation in expression of the following genes, Bcl-2 associated athanogene 3 (BAG-3) (4-fold), DNA-PKcs (3.6-fold) and Hsp70 (2.7-fold), at 48 hours suggests that the K1 ES cells may be undergoing some form of cellular stress.

BAG-3 belongs to a family of proteins which participate in a wide variety of cellular processes including cell survival (stress response) and apoptosis (Doong *et al.*, 2002). Over-expression of BAG-family proteins is found in several cancers, and has been demonstrated to enhance cell survival and proliferation

(Liao *et al.*, 2001). Each member contains at least one evolutionary conversed domain, a BAG domain (Doong *et al.*, 2002). The anti-apoptotic activities of BAG-family proteins are dependent on their interactions with the heat shock protein 70 or binding to Bcl-2. Heat shock proteins are a highly conserved family of molecular chaperones with diverse functions, including mediating protein folding and degradation, transport across cellular membranes, and assembly into macromolecular structures (Maheswaran *et al.*, 1998). HSP-70 and its constitutive form, HSC-70, are molecular chaperones activated by varied cellular stresses (For review see Hartle, 1996). DNA-PKcs is a DNA-dependent protein kinase (DNA-PK) that is essential for the repair of DNA double-strand breaks that form in cells that have undergone some form of stress e.g irradiation (For review see Lee and Kim, 2002).

Nedd4 and annexin XI are also differentially expressed between the E14 and K1 ES clones. Nedd4 (Neural precursor cell Expressed Developmentally Downregulated) was down-regulated 3.2-fold at 48 hours and up-regulated by 6-fold following 96 hours of treatment with RA in the K1 ES cell. Nedd4 was originally identified as being down-regulated during development of the mouse brain (Kumar *et* al., 1992). Subsequently, it has been demonstrated that Nedd4 is expressed in many other tissues and cell types (Kumar *et al.*, 1997). The *Nedd4* gene encodes an evolutionarily conserved ubiquitin-protein ligase. In addition to an ubiquitin-protein ligase domain, the Nedd4 protein contains a multiple WW domain and a calcium/lipid domain (Kanelis *et al.*, 2001). Through its WW domain, Nedd4 has been shown to interact with the epithelial sodium channel subunits, the haematopoetic transcription factor p45 and RNA polymerase II (Gavva *et al.*, 1997). Its interaction with these proteins partners probably mediates their turnover via ubiquitin-mediated pathways. Therefore, the role of Nedd4 may be to regulate a number of cellular proteins through ubiquitination. It has also been reported that Nedd4 is specifically cleaved by caspases during apoptosis (Harvey *et al.*, 1998). The significance of this

cleavage during apoptosis is unclear, however the function of Nedd4 may be required for the regulation of a protein(s) that are required for apoptosis.

Annexin X1 was up-regulated at 48 hours and also 96 hours in the K1 clone. An early event in apoptosis is the loss of membrane asymmetry of phospholipids (For review see Hacker, 2000). Annexin XI belongs a family of calcium-dependent phospholipid-binding proteins. Annexins are commonly used to detect apoptosis in cells, and therefore the up-regulation of this transcript may indicate the onset of apoptosis.

Therefore, in the K1 clones there is an increase in transcripts involved in cell survival, and also transcripts that may indicate the onset of apoptosis.

5.5.4.2 Retinoid Synthesis

WT1 null ES cells display differential expression of two genes involved in the *in vivo* regulation of retinoic acid synthesis. Cellular retinoic acid binding protein II (CRABP-II) was up-regulated 3.7-fold in the K1 clone at 96 hours. Cellular retinoic acid-binding proteins, CRABPs, are encoded by two distinct transcriptionally regulated genes that produce two highly conserved proteins, type I and II CRABP (CRABP-I and II) (Ruberte *et al.*, 1992; Lampron *et al.*, 1995). These two proteins differ in their tissue expression, as well as in ligand binding affinities. CRABP-II is expressed in the developing kidney, brain, spinal cord, stomach and limbs (Ruberte *et al.*, 1992; Ruberte *et al.*, 1993). In the kidney, at E10.5, CRABP-II is expressed in the developing mesonephros, whilst at E12.5, it is expressed in the developing metanephros. By E14.5 it can be detected in both the developing nephrons and surrounding stromal cells (Ruberte *et al.*, 1992). In the developing spinal cord at E10.5, CRABP-II was detected in the neuroepithelium and also in the differentiating motor neurons (Ruberte *et al.*, 1993). It has been proposed that CRABPs have multiple RA-related functions, including sequestering RA to lower functional intracellular

pools, transporting RA to nuclear receptors, and mediating RA metabolism to lower RA levels below teratogenic concentrations (Lampron *et al.*, 1995; Ruberte *et al.*, 1992). However, the actual function of CRABPs remains unclear as the double mouse knockouts of CRABP-I and CRABP-II are normal, indicating that CRABPs are dispensable *in vivo* for the control/modulation of RA during murine embryogenesis (Lampron *et al.*, 1995). In relation to WT1 and Wilms' tumour biology, in the analysis of gene expression in Wilms' tumours with Affymetrix Gene chip arrays, identified CRABP-II as being up-regulated (Li *et al.*, 2002).

RALDH-2 was down-regulated by 2.7-fold at 48 hours and 3-fold at 96 hours in the K1 ES cell clone. The *RALDH2* gene encodes a protein that is crucial for the *in vivo* synthesis of RA. Retinoic acid, the active derivative of vitamin A (retinol) is generated through two steps. The first step is the oxidisation of retinol to retinaldehyde, which is carried out by alcohol dehydrogenases (ADH1, ADH3 and ADH4). The second step is the oxidation of retinaldehyde to RA, which is carried out by retinaldehyde dehydrogenases (RALDH1, RALDH2 and RALDH3). Genetic analysis of enzymes regulating RA synthesis indicates that retinol is ubiquitously oxidised to retinal by alcohol dehydrogenases (Molotkov *et al.*, 2002), whilst retinal is tissue-specifically oxidised to RA by retinaldehyde dehydrogenases (Niederreither *et al.*, 1999). RALDH2 has been shown by *in situ* hybridisations and enzymatic studies to be the prominent RALDH involved in early mouse embryogenesis (Niederreither *et al.*, 1997; McCaffery *et al.*, 1995). RALDH2 is expressed from around E7.5 in trunk mesoderm, with additional expression by E8.5 in the optic vesicles (Niederreither *et al.*, 1997). Other sites of expression for RALDH2 is primarily in the mesodermal derivatives including somites, flank mesoderm, posterior hindbrain mesenchyme, and posterior heart mesoderm (Niederreither *et al.*, 1997; Niederreither *et al.*, 2002). Expression is also present in the nephrogenic zone of the kidney, and also in the motor neurons of the spinal cord (Sockanathan and Jessell, 1998; Haselbeck *et al.*, 1999; Niederreither *et al.*,

2002). Knockout of the RALDH2 gene results in early embryonic lethality (E9.5-E10.5), with the ablation of RALHD2 resulting in an almost total lack of retinoic acid synthesis (Niederreither *et al.*, 1999). RALDH2 knockout mice exhibit major patterning defects along the anteroposterior body axis and developing hindbrain, and also defects in the development of the heart (Niederreither *et al.*, 1999; Niederreither *et al.*, 2001).

5.5.4.3 IGFBPs and the CCN Family of Proteins

IGFBP-5 showed a 3.7-fold down-regulation in expression in the WT1 null ES cells at 48 hours and a 3.2-fold down-regulation at 96 hours. IGFBP-5 belongs to the Insulin Like Growth Factor Binding Protein (IGFBP) family. Insulin-like growth factors (IGFs) play a pivotal role in tissue homeostasis, regulating cell proliferation, differentiation and migration during development and in the adult. The actions of IGFs are modulated by high affinity interactions with a family of structurally related IGF-binding proteins, IGFBP-1 to IGFBP-6 (For review see Jones and Clemmons, 1995). IGFBPs are known to regulate the bioavailability of IGFs in the circulation; however, their functions at the cellular level are not fully understood. IGFBPs have been reported to both inhibit and enhance IGF-I action depending on the system employed (For review see Jones and Clemmons, 1995). IGFBP-5 has been shown to be involved in the regulation of several physiological processes in the ovary and kidney (Kelley *et al.*, 1996). Particularly in the kidney, IGFBP-5 was isolated in a screen for genes changing during mesenchymal-epithelial transition in the developing metanephric mesenchyme, a process characterised by up-regulation of WT1 (Plisov *et al.*, 2000). During this developmental process, IGFBP-5 expression increased. Also, the screening of gene expression in Wilms' tumours containing decreased WT1 exon 5 +/- isoform ratios with cDNA macroarrays demonstrated that IGFBP-5 was down-regulated (Baudry *et al.*, 2002). In the ES cell differentiation system, loss-of-function for WT1 results in a decrease in expression of IGFBP-5. Therefore, this correlates well with a potential regulation of IGFBP-5 by WT1.

WISP-1 was down-regulated 2.8-fold at 48 hours. WISP-1 is a member of the CCN family, which also includes connective tissue growth factor (CTGF), Cyr61 and Neproblastomas overexpressed (Nov). These proteins are cysteine-rich, secreted proteins associated with the extracellular matrix. Several members of the CCN family have been shown to support cell adhesion, induce focal adhesion complexes and stimulate adhesive signalling (Kireeva *et al.*, 1996; Chen *et al.*, 2001). One of the features of the CCN family of proteins is that all contain a domain that has homology to insulin-like growth factor binding proteins (IGFBPs). WISP-1 (Wnt-1 induced secreted protein 1) was originally identified in a differential screen for genes that were induced by Wnt-1 (Pennica *et al.*, 1998). Subsequently, it has been shown that WISP-1 is a β-catenin-regulated oncogene that can contribute to tumourigenesis (Xu *et al.*, 2000). Over-expression of WISP-1 in a normal rat kidney fibroblast cell line results in morphological transformation, accelerated cell growth and enhanced saturation density. When these WISP-1 over-expressing cells were injected into nude mice, they readily formed tumours (Xu *et al.*, 2000). WISP-1 has also been shown to be aberrantly expressed in both mammary tumours and colon cancer (Pennica *et al.*, 1998). Interestingly two other members of the CCN family, Nov and CTGF, have both been reported to be regulated by WT1 (Martinerie *et al.*, 1996; Stanhope-Baker and Williams, 2000).

5.5.4.4 Genes Involved in Kidney Development

There was an 8-fold reduction in the levels of Wnt-4 at 96 hours in the RA treated K1 ES cells. The *Wnt-4* gene encodes a protein belonging to the *Wnt* gene family of secreted glycoproteins. Wnt-4 is expressed in the metanephric mesenchyme of the kidney and its derivatives (Stark *et al.*, 1994). Expression is initiated in the aggregating mesenchyme that surrounds the invading ureteric bud at E11.5. Wnt-4 expression is maintained in the comma-shaped bodies, but later becomes restricted to the S-shaped bodies. There is also expression in domains outside the kidney, including the dorsal spinal cord, floor plate, pituitary gland, mammary gland and female gonads (Parr *et al.*, 1993; Vainio *et*

al., 1999). Deficiency in Wnt-4 results in abnormal development of the kidney, pituitary gland, female reproductive system, and mammary gland (Stark *et al.*, 1994; Vainio *et al.*, 1999; Heikkila *et al.*, 2002). The finding that Wnt-4 expression decreases on loss-of-WT1 is particularly interesting because Wnt-4 is believed to be involved in regulating the mesenchymal-epithelial transition involved in nephron differentiation, and this process fails in Wnt-4 null mice (Stark *et al.*, 1994). Recently, evidence for Wnt-4 being a potential WT1 target gene has been described using transfection of a dominant-negative WT1 variant into the M15 cells (Sim *et al.*, 2002).

Brain factor-2 (BF-2) or FoxD1 demonstrated a 3.7-fold reduction in expression in the K1 ES cell at 96 hours. BF-2 belongs to the family of winged helix transcription factors, which all share an evolutionarily conserved DNA binding domain (Lai *et al.*, 1993). In the mouse, BF-2 has a neuronal and mesenchymal expression pattern. In the nervous system, BF-2 is expressed predominantly in the diencephalon (Hatini *et al.*, 1994). Mesenchymal expression is detected in the mesenchyme of the head, the metanephric mesenchyme of the developing kidney and also cortex of the adrenal gland (Hatini *et al.*, 1994). Expression has also been reported in the testis (Pierrou *et al.*, 1994). In the kidney, BF-2 is first expressed in E11.5 metanephric mesenchyme. Its expression marks a population of cells that are peripheral to the condensing mesenchyme around the tips of the invading ureteric bud that gives rise to nephrogenic lineages. Therefore, BF-2 expression indicates an early step in the partitioning of the metanephric mesenchyme into stromal and nephrogenic lineages. A mouse knockout of BF-2 has been generated and there are defects observed in two components of the kidney; the ureteric bud epithelium and the nephrogenic mesenchyme (Hatini *et al.*, 1996). The ureter and the collecting system are smaller and the differentiation of the metanephric mesenchyme to nephrons is inhibited. The kidneys of BF-2 null mice are hypoplastic, fused longitudinally and exhibit short ureters (Hatini *et al.*, 1996). They also exhibit decreased branching morphogenesis and are arrested in the mesenchymal-epithelial transition, with

large mesenchymal aggregates present. Given that BF-2 is not expressed in the nephrogenic mesenchyme of the kidney, nor in the epithelia of the nephron intermediates, it seems likely that the BF-2 transcription factor regulates the expression of a stromal secreted molecule which in turn regulates the differentiation of the nephrogenic lineages.

Sprouty-4 demonstrates 3.3-fold down-regulation in the WT1 null ES cells after 48 hours. The first *Sprouty* gene was identified in *Drosophila* as a regulator of branching morphogenesis in the trachea, where it acts as an antagonist to fibroblast growth factor signalling pathways (Hacohen *et al.*, 1998). Further analysis has shown that it acts as an antagonist of epidermal growth factor, as well as the FGF signalling pathway (Reich *et al.*, 1999). To date, four *Sprouty* genes have been identified in mouse, with each shown to possess distinctive overlapping expression patterns (Minowada *et al.*, 1999; Zhang et al., 2001). Sprouty-4 has a mainly neuronal and mesenchymal expression pattern, with expression detected in the cerebellum, spinal cord and ganglia, lung, nasal mesenchyme, digestive tract, limb buds and kidney (Minowada *et al.*, 1999; Zhang *et al.*, 2001). In the developing kidney, Sprouty-4 is expressed in the ureteric bud and the metanephric mesenchyme at around E10.5, whilst at E14.5 it is expressed in the developing glomeruli (Zhang et al., 2001).

5.5.4.5 Other Transcripts

Peripherin was represented twice on the Affymetrix Genechip array, once as an annotated transcript, the other as an EST entry. At 48 hours, both entries for peripherin demonstrated similar values of fold change, with a 3.3-fold and 3.1-fold up-regulation in the K1 ES clone. At 96 hours both entries for peripherin also show an up-regulation, with a 4.3-fold and 9.1-fold change. Peripherin is a type III neuron specific intermediate filament protein, that is primarily expressed in the peripheral nervous system and only at low levels in defined neuronal populations of the central nervous system (Troy *et al.*, 1990; Escurat *et al.*, 1990). Peripherin expression is first detected at E9 in the mouse, with

expression detected in the myelencephalon (Troy *et al.*, 1990). Between E9.5-E12 of mouse embryogenesis, expression of peripherin can be detected in the developing motor neurons of the ventral neural tube and also the developing dorsal root ganglia (Troy *et al.*, 1990). In Chapter 4, WT1 expression was detected in the developing motor neurons in the neural tube, and therefore WT1 may act to regulate its expression in these domains.

CYP1B1 was down-regulated 3.7-fold at 48 hours and 3.2-fold at 96 hours in the K1 ES cells treated with RA. CYP1B1 is a member of the cytochrome P450 multigene superfamily of proteins, which are enzymes involved in the metabolism of a wide range of structurally diverse substrates (For review see Stoilov *et al.*, 2001). Cytochrome P450 (CYP) proteins are ubiquitous in nature, appearing in almost all phyla, with each isoform exhibiting distinct temporal and spatial expression patterns during embryogenesis (Stoilov *et al.*, 2001). From the study of numerous identified isoforms of cytochrome P450 during embryogenesis, it has been proposed that their function is to serve as facilitators of morphogenesis in the embryonic tissues (Stoilov *et al.*, 2001). The cytochrome P450 isoforms may either function to generate morphogenic molecules or to keep regions free of them, thereby creating temporal and spatial regions of morphogen action to support region-specific changes in cells.

CYP1B1 has a pattern of expression that differs from the other two CYP1 family P450s (CYP1A1 and CYP1A2); it is expressed constitutively in steroidogenic tissues like the adrenal gland, ovary, and testes, and is inducible by adrenocorticotropin, cAMP, peptide hormones, and aryl hydrocarbon receptor ligands (Zhang *et al.*, 1998; Buters *et al.*, 1999). CYP1B1 is also expressed in steroid-responsive tissue such as the uterus, kidney, breast, and prostrate (Savas *et al.*, 1994; Buters *et al.*, 1999). The conservation of CYP1B1 and its expression in reproductive tissue would suggest that it has an important developmental or physiological role in mammals. However, the precise role of CYP1B1 in fertility and reproduction is unclear as a mouse knockout of

CYP1B1 has no gross phenotype and homozygous CYP1B1 null mice develop normally and are fertile (Buters *et al.*, 1999).

Mutations in the *CYP1B1* gene have been demonstrated to be the common cause of human primary congenital glaucoma (Stoilov *et al.*, 1997). These mutations are thought to generate a null allele of *CYP1B1*. Primary congenital glaucoma (PCG) is an autosomal recessive eye disorder that is believed to arise from development defects in the anterior eye segment. Closer analysis of the CYP1B1 knockout mice demonstrates that the CYP1B1$^{-/-}$ mice have ocular drainage structure abnormalities resembling those reported in human PCG patients (Libby *et al.*, 2003). The possible regulation of CYP1B1 by WT1, and its link to ocular disorders, is intriguing, given that patients with Wilms' tumour are at an increased risk for developing ocular disorders including aniridia and, less frequently, optic nerve hypoplasia (Nelson *et al.*, 1984; Bickmore and Hastie, 1989). These eye abnormalities in Wilms' tumour patients are thought to result from concomitant disruption of the aniridia gene PAX6, which lies telomeric of WT1 on human chromosome 11p13 (Ton *et al.*, 1991). However ocular defects have been recently described in the WT1 null mice (Wagner *et al.*, 2002).

5.5.6 Verification of Microarrays

Examining the list of differentially expressed genes that were down-regulated in the K1 clone array, Wnt-4 and BF-2 were chosen to verify the finding of the microarrays. Wnt-4 was down-regulated 8-fold in the K1 clone, whilst BF-2 exhibited a 3.7-fold reduction in levels of expression compared to the wild-type ES cell after 96 hours. Semi-quantitative RT-PCR was carried out for BF-2 and Wnt-4 on replicate samples of RNA collected from the original differentiation of E14 and K1 ES cells described in section 5.4. Also, to ensure that the down-regulation of BF-2 and Wnt-4 was not a clonal artifact, RNA was collected from the differentiation of the second WT1 null clone, K46, following 96 hours with RA, along with RNA collected from an independent differentiation of another

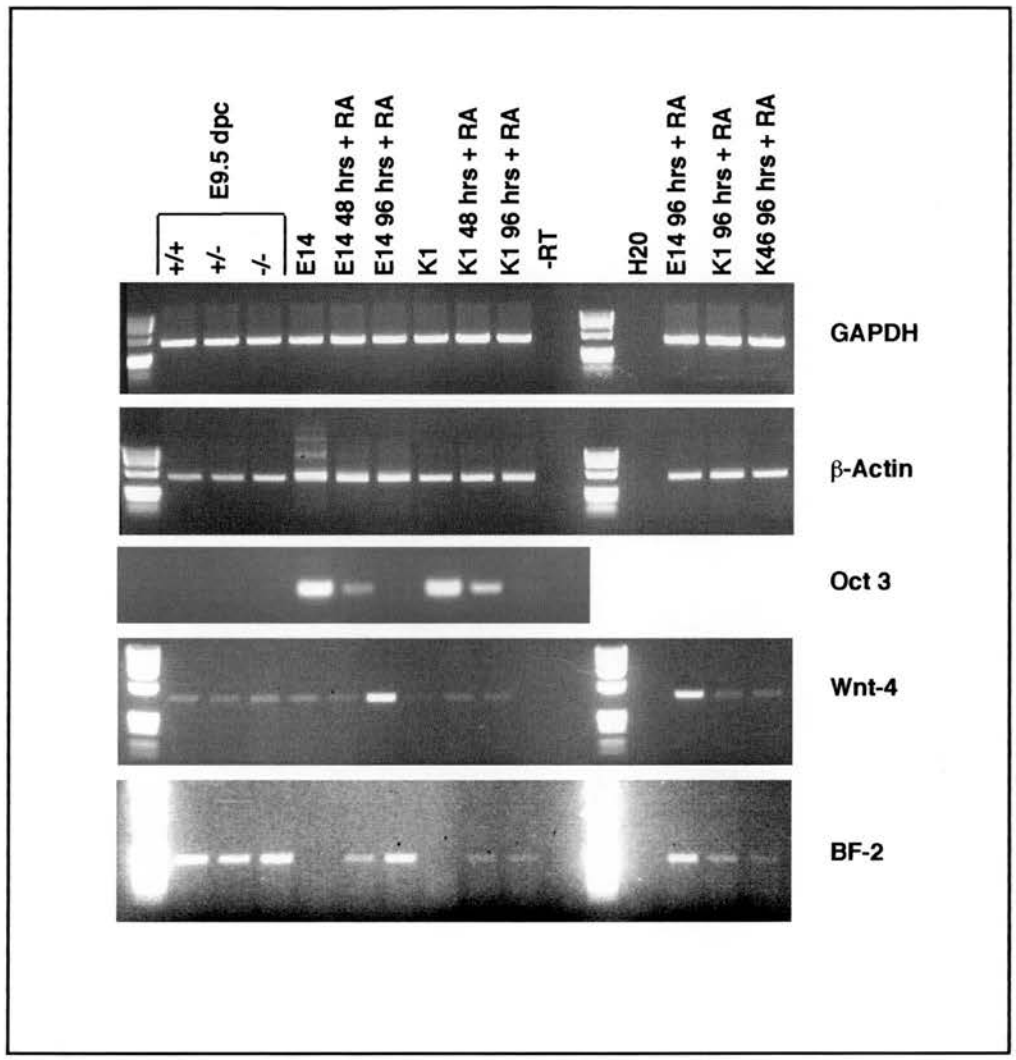


Figure 5.16 Semi-Quantitative RT-PCR Confirms Differential Expression of BF-2 and Wnt-4 Transcripts in the RA Treated WT1 Null ES Clones

Total RNA was collected from wild-type, WT1 heterozygote and WT1null whole E9.5 embryos, and from replicate experiments of RA treated E14 and K1 ES clones. Total RNA from an independent RA differentiation of E14 and K1, in addition to the second WT1 null ES cell line, K46, was also collected. 5 μg of RNA was reverse transcribed to produce cDNA. To control for the levels of cDNA, the samples were normalised with respect to the expression levels of β-actin and GAPDH (26 cycles). RT-PCR for Wnt-4 (27 cycles) and BF-2 (26 cycles) was carried out.

passage of E14 and K1 ES cells. By semi-quantitative RT-PCR, both BF-2 and Wnt-4 were down-regulated in both the replicate sample of K1 RNA and also in the K46 RNA samples at 96 hours (Figure 5.16). This down-regulation therefore confirms the findings of the microarray, and also confirms that the down-regulation of gene expression is not due to clonal variation.

Whether the transcripts differentially expressed between E14 and K1 during the differentiation of the ES clones are due to direct regulation by WT1 is difficult to ascertain at this point. However, it can be established whether or not the expression of WT1 and a certain potential target are cell specific, in that they are both expressed in the same cell populations. If the cells co-express both WT1 and the transcript of interest, then this might add further evidence to indicate that the regulation of the transcript by WT1 maybe be cell specific and direct, and not through an indirect process such as a paracrine/autocrine loop. From the array data, Wnt-4 represented the highest fold change across the two time points, with an 8-fold reduction in expression in the K1 sample compared to the E14 sample at 96 hours. Given its relevance physiologically and also the recent data indicating that this might be a target of WT1 (Sim *et al.*, 2002), the expression of Wnt-4 in the wild-type differentiated ES cells was monitored by immunofluorescence to see whether Wnt-4 is co-expressed with WT1. Wild-type ES cells were co-stained with WT1 and Wnt-4 in an undifferentiated state and following 96 hours of treatment with RA (Figure 5.17). In the undifferentiated state there was no detection of either Wnt-4 or WT1. After 96 hours of differentiation, WT1 staining can be detected in approximately 75-80% of cells. In differentiated ES cells with high levels of WT1 expression, there is co-expression of Wnt-4 (Figure 5.17 (b) and Figure 5.17 (c)). The staining pattern for Wnt-4 is distinctive and appears to localise Wnt-4 to the endoplasmic reticulum or the golgi apparatus of the cells. This is consistent with the previous report of localisation of transiently expressed Wnt proteins in COS cells where antibody staining demonstrated that all Wnt proteins were primarily retained in the endoplasmic reticulum (Burrus and McMahon, 1995).

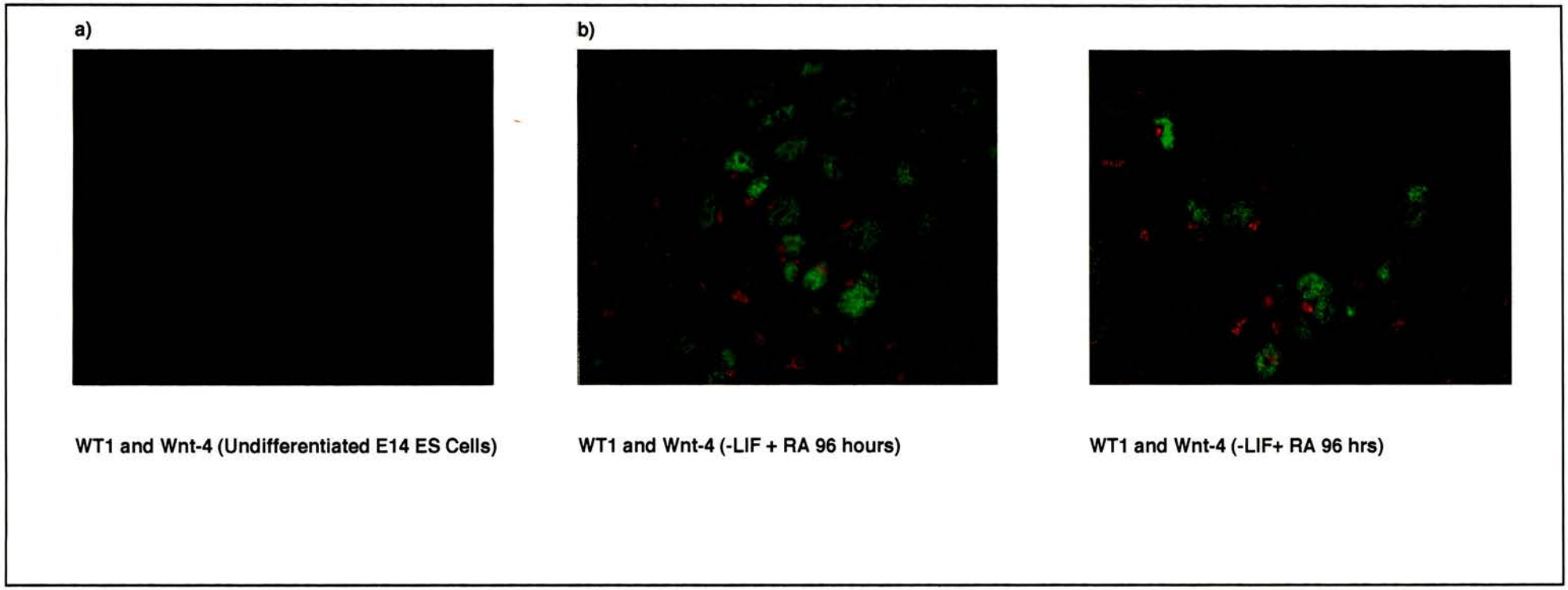


Figure 5.17 WT1 and Wnt-4 are Co-Expressed in E14 ES Monolayer Cultures DIfferentiated for 96 hours with RA.

a) Undifferentiated E14 ES cells and b) E14 ES cells treated with RA for 96 hours, were co-stainied for WT1(green) and Wnt-4 (red). (Magnification x 20)

5.5.7 Published WT1 Target Genes

Next the expression profiles of previously reported candidate target genes were analysed to determine whether their expression was altered in the K1 ES cells after 48 hours and 96 hours in comparison to E14 cells at the same time points (Figure 5.18). Amphiregulin, BF-2, and Wnt-4 were the only previously reported candidate WT1 target genes that were differentially expressed more than the 2.5-fold value (Lee *et al.*, 1999; Ernstsson *et al.*, 1996; Sim *et al.*, 2002). However changes were seen in the system for some of the putative WT1 targets below the 2.5-fold change.

A 2.2-2.5-fold induction of c-Myc expression in the K1 clone from 48-96 hours is observed, consistent with the published transcriptional repression of the *c-Myc* gene by WT1 (Hewitt *et al.*, 1995). IGFBP-4 was identified as being down-regulated in ES cells that were homozygous for a truncated form of WT1 (Wagner *et al.*, 2001). In this study the similar down-regulation of IGFBP-4 observed (2.3-fold), adds support to the possibility that IGFBP-4 is regulated by WT1. However there was no change in cyclin G1, another gene identified as being down-regulated in the RA treated DDS ES cell lines (Wagner *et al.*, 2001).

Some of the WT1 target genes do not respond in this system in a manner that was originally described. For example, amphiregulin was identified as transcriptionally activated by WT1 (Lee *et al.*, 2001). Therefore, in comparing the E14 and K1 expression profile, one might expect a reduction in the levels of amphiregulin in the K1 samples. However there is a 3-fold increase in amphiregulin expression in the K1 sample. The same phenomenon is observed when expression levels of CTGF are compared. CTGF was identified from gene expression profiles of a Wilms' tumour cell line stably expressing aDDS-WT1 construct (Stanhope-Baker and Williams, 2000). In this system, CTGF was up-regulated in the DDS-Wilms' tumour cell lines. *In vitro* luciferase assays

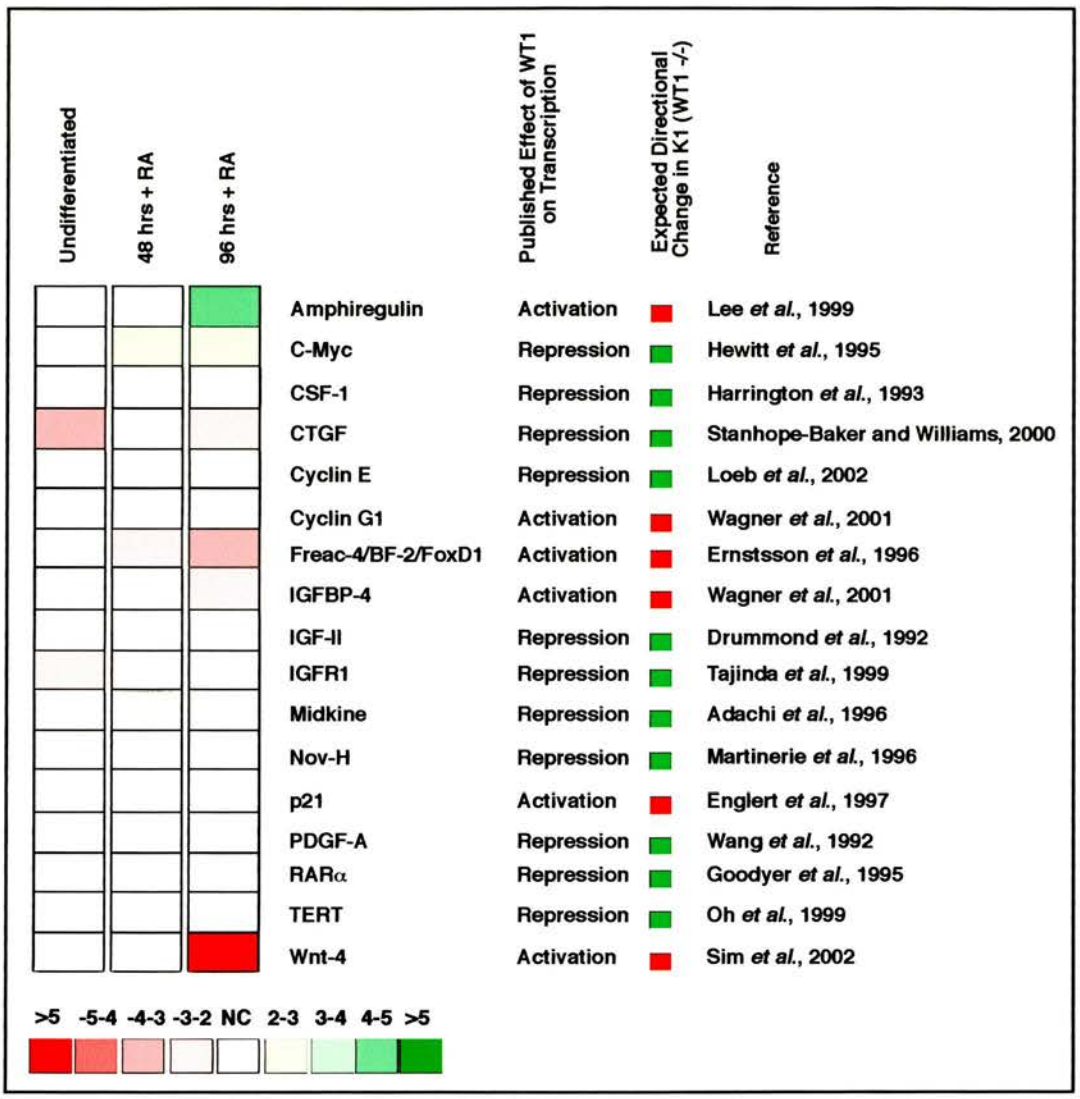


Figure 5.18 The Expression Profile of Published WT1 Target Genes in the K1 Clone in an Undifferentiated State, Treated with RA for 48 hours and Treated with RA for 96 hours.

Published WT1 target genes, which were present on the Affymetrix Mouse Gene Chip U74A array, were analysed to see if any exhibited differential expression between E14 and K1 ES cells at the three time points. The published effect of WT1 upon the behaviour of the target gene is shown, along with the expected directional change of gene expression that would be present in the K1 clone in comparison to the E14 ES cells.

demonstrated that the CTGF promoter was repressed by WT1. Therefore, one might expect that in the K1 sample there would be an up-regulation of CTGF, however CTGF is down-regulated by 2-fold in the K1 sample compared to the E14 sample. Some caution must be observed in the analysis of the effects of WT1 loss-of-function on the expression of CTGF, as CTGF was down-regulated 3-fold in the K1 ES clone in the undifferentiated state compared to the undifferentiated E14 ES cells. Overall, the differences observed in the behaviour of amphiregulin and CTGF, and the lack of confirmation of other published putative WT1 targets, may indicate differences in the cell system used in this study and the system used to originally identify them as putative WT1 target genes.

5.6 Discussion

The generation of the WT1 null ES cells in this chapter has provided an important resource to further investigate the role of WT1 in the molecular control of cellular differentiation. From the use of the RA differentiation protocol, in conjunction with the Affymetrix Genechip microarrays, has pointed towards various cellular changes that are occurring following WT1 loss-of-function in K1 cells during RA differentiation, and also identified a set of novel candidate WT1 target genes.

The initial aim of the project was to generate WT1 null ES cells using the conditional targeting vector described in Chapter 3. In employing this targeting vector, correct homologous targeting of the remaining wild-type allele would have generated a heterozygote clone in which complete ablation of WT1 would have been under the temporal control of *Cre* recombinase. However attempts to generate a conditional null ES cell line proved difficult due to problems associated with the targeting of the remaining wild-type allele with the *pWT1LoxP* targeting vector. One clone was obtained in which the targeting vector was partially incorporated into the *WT1* endogenous locus, but with the

5' end of the vector (containing the final *loxP* site) absent. The failure to incorporate the 5' *loxP* site into the locus could reflect the fact that the selection cassette is approximately 4 kb away from this region and therefore is under reduced selective pressure. The relative ease in which the second allele was successfully targeted with the *pWT1Kre* targeting vector may be due to the fact that this targeting vector has longer arms of homology to WT1, and is not dependent upon the insertion of a 34 bp *loxP* site in the 5' region. The successful generation of a correctly targeted ES cell lines with the *pWT1Kre* construct, and the demonstration that these clones were WT1 null ES cells, indicates that the *Cre/LoxP* inactivation of the first allele does produce a true null allele. This is crucial as it demonstrates the *Cre/LoxP* modifications to the *WT1* locus will produce a *WT1* null allele *in vivo*.

Examination of the WT1 null ES cell lines in an undifferentiated state indicated that the WT1 null ES cells were phenotypically similar to wild-type ES cells. In the analysis of WT1 null ES cells during treatment with RA, morphologically the cells appeared to be comparable, however there were differences in the cell number after 48 hours. At 72 hours of treatment with RA, there was a 50% reduction in WT1 null ES cells in comparison to wild-type controls. Whether this reduction in cell numbers is due to apoptosis or a decrease in proliferation is unknown. However, it is clear that this event was specific for the absence of WT1, as WT1 heterozygote null ES cells behaved in a similar manner to wild-type ES cells when treated with RA. This therefore provides an excellent system to study the role of WT1 in the regulation of proliferation and/or apoptosis..

A similar response was seen in myeloid leukaemia cell lines in which WT1 was "knocked down" using WT1 antisense oligonucleotides that were targeted to the translation initiation site of WT1 (Algar *et al.*, 1996). In cell lines treated with the WT1 antisense oligonucleotide, there was a significant reduction in cellular proliferation and increase in apoptosis. The authors suggested that was necessary for cell proliferation and maintaining viability in these cell lines. A

similar scenerio is observed in the metanephric mesenchyme of the kidney, where in the WT1 null embryo the absence of WT1 results in apoptosis of the metanephric mesenchyme. Therefore, could the expression of WT1 be necessary for maintaining the viability of some lineages that are generated in the RA treatment of the WT1 null ES cells? This appears to be unlikely, as the analysis of gene expression at 96 hours indicates that there is not a radical shift in the expression profiles between the wild-type and WT1 null ES cells (Discussed below).

In Chapter 4, a limited analysis of E14 differentiated ES cells by a selection of antibodies and RT-PCR analysis demonstrated the presence of neuronal lineages, and the absence of mesodermal lineages, within the monolayer cultures following 96 hours of treatment with RA. The Affymetrix Genechip array analysis of the differentiated wild-type E14 ES cells confirms some of these findings. However, it also suggests that there maybe other lineages arising from the RA differentiation of the cells. Interestingly, the analysis of transcripts that are down-regulated when E14 ES cells are treated with RA has identified several genes and ESTs that might be involved in maintaining ES pluripotency.

Affymetrix Genechip arrays containing 12,000 transcripts were screened to identify novel candidate WT1 target genes in RA differentiating ES cells. Overall the ablation of WT1 appears not to have induced massive differences in gene expression at the time points that were chosen. This is demonstrated by the analysis of the gene expression profiles by scatter plot analysis and confirmed by the fact that across the two time points, there were in total only 102 transcripts that were differentially expressed greater than 2.5-fold between the wild-type and WT1 null ES cells. Within this set of differentially expressed transcripts, several are present that have been previously reported in other systems to represent WT1 downstream targets, notably amphiregulin and Wnt-4 (Lee *et al.*, 1999; Sim *et al.*, 2002). Although amphiregulin has been reported to be a direct target for WT1, in the ES cell differentiation system described in this

chapter, the loss of WT1 leads to an increase in the expression levels of amphiregulin, an effect that is in contradiction to the reported transcriptional activation of amphiregulin by WT1. However the dramatic down-regulation of Wnt-4 in the WT1 null ES cells is consistent with the previous reports of Wnt-4 regulation by WT1 (Sim *et al.*, 2002), and further supports the notion that Wnt-4 is directly regulated by WT1. Further evidence that WT1 directly regulates Wnt-4 expression has come from work using RNA interference (RNAi) in mesonephric cell lines and kidney organ cultures. Knockdown of WT1 by RNAi in the M15 cell lines results in a down-regulation of Wnt-4 (Davies, Ladomery, Hohenstein, Michael, Spraggon, Freeman and Hastie, 2003, Manuscript submitted).

The human homologue of *BF-2*, *FREAC-4*, has previously been shown be a potential WT1 target gene (Ernstsson *et al.*, 1996). Using transient transfections, a construct containing 2 kb of genomic sequence upstream of the *FREAC-4* start site, linked to a luciferase reporter, demonstrated a 3-fold induction when co-transfected with a WT1 expression plasmid into a kidney derived cell line (Ernstsson *et al.*, 1996). Transfections of 5' deletions of the 2 kb *FREAC-4* luceriferase construct, followed by gel shift assays using recombinant WT1, identified a WT1 binding site within the *FREAC-4* promoter (Ernstsson *et al.*, 1996). In the ES differentiation system, the loss WT1 results in a down-regulation of the murine homologue of *FREAC-4*, BF-2. From the results presented in this chapter, and also the previous work using transient transfections of reporter constructs of human homologue of *BF-2*, *FREAC-4*, it suggests that WT1 may activate the expression of BF-2/FREAC-4. However, it's difficult to tie this into the *in vivo* situation with regards to WT1 regulation of BF-2. It is clear that BF-2 and WT1 do have overlapping expression patterns during embryogenesis, with expression in the metanephric kidney, testis and CNS. It has already been demonstrated that BF-2 is necessary for normal development, not only of the stroma but also of the nephrogenic lineages, suggesting that it regulates stromal production of diffusible regulators of

nephron differentiation. Loss of BF-2 results in the appearance of aggregates of proliferating, poorly-differentiated mesenchyme in place of where nephrons would normally be expected to form, a similar phenotype noted in the Wnt-4 knockout mice (Stark *et al.*, 1994). Although it is unclear whether stromal cells and metanephric mesenchyme originate from a common precursor or from distinct lineages, stromal cell progenitors can be distinguished from epithelial progenitors at the earliest stages of renal morphogenesis (Hatini *et al.*, 1996), consistent with the proposed role of stroma as an essential and independent component of the reciprocal signalling pathways. Since WT1 expression precedes that of BF-2 in the metanephric mesenchyme, it is possible that during these early stages WT1 activates BF-2 expression, which then proceeds to define the stromal component of the metanephric mesenchyme from the nephrogenic component.

From the analysis of gene expression profiles between wild-type and WT1 null ES cells, a novel set of candidate WT1 regulated genes has been identified. The potential regulation of RALDH2 by WT1 points to an intriguing possibility that WT1 may be involved in the regulation of the retinoid acid synthesis pathway. Retinoic acid has profound effects *in vivo* stimulating proliferation, differentiation and morphogenesis (For review see Mark *et al.*, 1999). A link for WT1 within the retinoic acid pathway has previously been reported, with WT1 shown to repress the retinoic acid receptor α gene (RAR-α) (Goodyer *et al.*, 1995). However this study was carried out using transient transfection, deletion analysis and EMSA, and hence the physiological significance of this regulation has yet to be established. Also RAR-α possesses a ubiquitous expression pattern (Dolle *et al.*, 1990), whilst WT1 is tissue specific. In comparison, RALDH2 is highly tissue specific and does have an overlapping expression pattern with that of WT1. In the epicardium and the epicardially derived cells of the heart, WT1 and RALDH2 are co-expressed (Perez-Pomares *et al.*, 2002). Reducing/abolishing the expression of WT1 within these cells, as is the case of the WT1 knockout mouse or in experimental perturbation of epicardial

development *in vitro*, has the effect of reducing the levels of RALDH2 (Perez-Pomares et al., 2002; Professor Munoz-Chapuli, personal communication). In Chapter 4, the observation that WT1 is expressed in motor neurons in the spinal cord is also a site for expression of RALDH2 (Sockanathan and Jessell, 1998; Haselbeck *et al.*, 1999; Niederreither *et al.*, 2002). In the context of the spinal cord, RALDH2 expression and the subsequent local generation of RA has been shown to specify the subtype identity of spinal motor neurons (Sockanathan *et al.*, 1998). It would be interesting to analyse whether the down-regulation of RALDH2 in the epicardium of the heart in the WT1 null embryos is mimicked in the spinal cord of WT1 null embryos. This may have implications for changes in subtype patterning in the spinal cord of the WT1 null embryos.

IGFBP-5 and WISP-1 represent another set of potentially interesting transcripts that were differentially expressed in the WT1 null ES cells. IGFBP-5 belongs to the family of Insulin like growth factor binding proteins (IGFBPs) which are a component of the insulin-like growth factor system. These proteins form high-affinity complexes with IGF-I and IGF-II, and thereby either inhibit or potentiate their effects *in vivo*. To date, the IGFBP family comprises 10 proteins that includes a superfamily of 6 proteins (IGFBP-1 to –6) which bind with high affinity and specificity to IGFs. Recently, a smaller family, the Insulin-like Growth Factor Binding Protein related peptides (IGFBPrp 1-4), that include IGFBP-7 and putative IGFBP-8, -9, and –10 that bind with a lower affinity, have been identified (Kim *et al.*, 1997, Ferry *et al.*, 1999). Previously, IGFBP-4 was identified as being differentially expressed in RA treated ES cells that contained a modification to the zinc finger domain of WT1 (Wagner *et al.*, 2001). In WT1 null ES cells, IGFBP-4 was similarly down-regulated, as was IGFBP-5. Therefore, WT1 may regulate some of the IGFBP proteins, and in doing so modulates the regulation of free levels of IGF-I and IGF-II. WISP-1 belongs to the CCN family, which includes connective tissue growth factor (CTGF) and Neproblastomas overexpressed (Nov). Both of these genes encode proteins which are structurally related to the IGFBP family of proteins. Also

CTGF and Nov have been reported previously to be regulated by WT1 (Stanhope-Baker and Williams, 2000; Martinerie *et al.*, 1996), and therefore WISP-1 is another member of the CNN family that might be regulated by WT1.

The Affymetrix Genechip arrays have proved an extremely powerful tool for the discovery of WT1 candidate target genes. However, it is important to address the limitations of the work presented in this chapter. The Affymetrix Genechip arrays have provided preliminary data sets. Replicate arrays will be required to confirm the findings presented in this thesis. Also, the results described in this chapter do not address the question of whether the effects observed in the gene expression profile are directly mediated by WT1, or indirectly mediated by another gene product. No single well-defined consensus sequence for promoter binding by WT1 has been identified. Sequences bound by WT1 include the WTE (5'-GCCTGGGAGT-3'), WRE (5'-GCGTGGGAGT-3') and EGR1 (5'-GCGGGGCG-3') (Lee and Haber, 2001). The analysis of the murine promoters of *Peripherin, CYP1B1, CRABP-II, RALHD2, IGFBP-5, WISP-1* and *Wnt-4* failed to detect the presence of a complete EGR1, WTE or WRE sites in the 1kb upstream promoter sequence of this genes (Data not shown).

In summary, the data presented in this chapter shows that ablation of WT1 in ES cells, coupled with Affymetrix Genechip arrays of gene expression profiles, is a useful approach which has identified a set of differentially expressed transcripts which may represent candidate WT1 regulated genes. Future work will be required to verify whether these transcripts are directly regulated by WT1, and also whether the mis-regulation of these transcripts has any effect on the pathological state of diseases, such as Wilms' tumours or diseases associated with loss-of-function mutations in WT1.

Chapter 6 Identification of a Novel WT1 Interacting Protein

Chapter 6 Identification of a Novel WT1 Interacting Protein

6.1 Introduction

The identification of additional components of the cellular machinery with which WT1 interacts will permit a greater understanding of the molecular pathways that WT1 is involved in. There are many examples of transcription factors interacting with other proteins to exert their function within the cell, one of the best being the GATA transcription factors.

6.1.1 GATA Transcription Factors & Friends of GATA (FOG) Proteins

The GATA family of zinc finger transcription factors are important regulators of development in *Caenorhabditis*, *Drosophila* and vertebrates. They have been shown to play a crucial role in various developmental processes including the differentiation of hematopoietic cells such as T cells, cardiac and coronary vasculature development and liver, lung and gut morphogenesis (Cantor and Orkin, 2002).

GATA transcription factors bind to a DNA consensus sequence of GATA. The transcriptional activities of the GATA proteins are modulated by interaction with both transcriptional co-activators and repressors. For example, the transcription factors SP1 and EKLF interact with the C-terminal zinc finger of GATA-1 to synergistically activate erythroid-specific gene expression (Fischer *et al.*, 1993; Merika & Orkin, 1995). Similarly Nkx2.5 associates with the C-terminal zinc finger of GATA-4 to activate the transcription of cardiac-restricted genes (Durocher *et al.*, 1997; Sepulveda *et al.*, 1998).

More recently, a family of multi-type zinc finger proteins has been identified which also interact with GATA factors. These zinc finger proteins belong to the FOG (Friends of GATA) family and include FOG1, FOG2, xFOG and USH (Tevosian *et al.*, 1999; Fossett *et al.*, 2001; Cantor & Orkin, 2001). An example of the importance of the interaction between GATA factors and FOG proteins has recently been demonstrated during gonadal differentiation. Direct association between GATA-4 and FOG2 is required for normal gonadal differentiation, sex determination and normal expression of the Sertoli-specific transcription factor SRY (Tevosian *et al.*, 2002). In mice homozygous null for either FOG2 or GATA-4, a direct association between the two proteins can not occur, leading to abnormalities during gonadogenesis. It was found that *Sry* transcript levels were significantly reduced in XY Fog2–/– gonads at E11.5, which is the time when Sry expression normally reaches its peak. In addition, three genes crucial for normal Sertoli cell function (Sox9, Mis and Dhh) and three leydig cell steroid biosynthetic enzymes (p450scc, 3ßHSD and p450c17) were not expressed in XY Fog2–/– and Gata4 –/– gonads, whereas Wnt-4, a gene required for normal ovarian development, was expressed ectopically (Tevosian *et al.*, 2002). Therefore the loss either the transcription factor itself, or its partner proteins, can have dramatic developmental and biological consequences.

Another example is the monocyte enhancer factor 2 proteins (MEF2). MEF2 proteins are MADS-box transcription factors that are essential for differentiation of all muscle lineages. In mammals, the MEF2 family is composed of four members: MEF2A; MEF2B; MEF2C; and MEF2D. The earliest site of MEF2 expression is within the heart, where expression of MEF2C is detected at E7.5. Inactivation of MEF2C in mice leads to severe cardiac developmental arrest and down-regulation of a number of cardiac markers. One of the cardiac genes shown to be down-regulated was αMHC (Lin *et al.*, 1997). Closer inspection of the αMHC promoter indicates the presence of two low-affinity MEF2 binding sites. However, *in vitro* co-transfection assays fail to show that MEF2 can

activate αMHC-driven reporters (Morin *et al.*, 2000). Subsequent work now indicates that the MEF2 proteins are recruited to the promoter region by the cardiac- specific transcription factor GATA-4, to synergistically activate αMHC, in addition to several other MEF2C target promoters (Morin *et al.*, 2000).

6.1.2 Published WT1 Interacting Proteins

A number of different approaches have been employed to identify potential protein partners of WT1. From these studies, proteins have been identified that either modulate the function of WT1 as a transcription factor, affect the activity of WT1 in cell cycle regulation and apoptosis, or which implicate a role for WT1 in RNA splicing and post-transcriptional regulation (Refer to Table 6.1).

6.1.2.1 Transcriptional Regulation

Using immunoprecipitations techniques, WT1 has been shown to interact with p53 (Masheswaran *et al.*, 1993). Immunoprecipitations for WT1 from transformed Baby Rat Kidney (BRK) cells and a Wilm's tumour sample, both of which express WT1, demonstrated that WT1 was associated with p53 (Masheswaran *et al.*, 1993). This protein-protein interaction was mediated through zinc fingers 1 and 2 of WT1. In a particular cellular context, the association of p53 with WT1 converts it from a transcriptional activator to a transcriptional repressor. Further work has established that the interaction of WT1 with p53 modulates the activity of p53 (Masheswaran *et al.*, 1995). In BRK cells stably transfected with WT1, an increased level of endogenous p53 protein was observed. This increase in the level of p53 protein was assumed to be due the increase of the steady state levels of p53, by prolonging the half-life of the p53 protein. The stabilised p53 demonstrated enhanced trans-activation properties. In addition, WT1 association with p53, inhibited p53-mediated apoptosis, but did not affect p53-mediated cell cycle arrest.

Recently, WT1 has been shown to interact with p73 and p63, two genes that share sequence homology with the transactivation, DNA binding and tetramerization domains of p53 (Kaghad *et al.*, 1997; Yang *et al.*, 1998; Scharnhorst *et al.*, 2000). In contrast to p53, which is dispensable for embryonic development, p73 and p63 are intimately involved in differentiation and development. P73 knockout mice exhibit profound neurological developmental defects, chronic infections and inflammation, as well as abnormalities in pheromone sensory pathways (Yang *et al.*, 2000). P63 knockout mice have absent or truncated limbs, defects that are caused by the failure of the apical ectodermal ridge to differentiate. Structures dependent upon epithelial-mesenchymal interactions during embryonic development, such as hair follicles, teeth and mammary glands, are absent in p63-deficient mice (Mills *et al.*, 1999).

The association of WT1 with p73 is mediated via the zinc fingers of WT1 and consequently, binding of WT1 to p73 diminishes DNA binding and transactivation by WT1. Similarly, WT1 inhibits p73-induced transcriptional activation in reporter assays and counteracts p73-induced expression of endogenous Mdm2. Overall the association between WT1 and the members of the p53 family of proteins may be an important determinant of their functions in cell growth and differentiation.

Ciao-1 was identified as a novel WT1-interacting protein which associates with the zinc finger domain (Johnstone *et al.*, 1998). GST-binding and co-immunoprecipitation of ectopically expressed WT1 and Ciao-1 demonstrated that a physical interaction occurs between these two proteins. Ciao-1 belongs to a family of proteins that contain WD40 or transducing repeats. Proteins containing WD40 repeats have several functions including cell cycle regulation, RNA splicing and transcription (Neer *et al.*, 1994). The significance of this interaction is unclear, although Ciao-1 may have a role in the modulating the transactivation activity of WT1.

Recently affinity chromatography of nuclear extracts from HeLa cells passed over immobilised WT1 protein, followed by mass spectrometric analysis of specifically retained proteins, identified Bone Marrow Zinc Finger 2 (BMZF2) as a protein which interacts with the zinc fingers of WT1 (Lee *et al.*, 2002). The function of this interaction appears to be to modulate the transactivation properties of WT1 resulting in a reduced transcriptional activity of WT1 (Lee *et al.*, 2002).

6.1.2.2 Cell Cycle Regulation

Other WT1 protein partners have been identified which regulate the effects of WT1 on cell cycle progression, to inhibit or induce apoptosis. WT1 has been shown to interact with the heat shock protein 70 (Hsp70) (Maheswaran *et al.*, 1998). This was demonstrated by establishing an osteosarcoma cell line with inducible, tetracycline-regulated WT1, coupled to immunoprecipitation of WT1 and microsequencing analysis of WT1 associated proteins. Hsp70 was identified in this screen, and further analysis showed that in fetal kidney Hsp70 was recruited to the characteristic subnuclear clusters that contain WT1. The domains of interaction with Hsp70 were shown to lie in the N-terminal region of WT1. The significance of this interaction is thought to modulate the growth-suppressive function of WT1. The N-terminal region of WT1 has been shown to be required for growth inhibition by WT1, which is mediated through the induction of the cyclin dependent kinase, p21, which leads to G1 phase cell cycle arrest (Englert *et al.*, 1997).

Prostrate Apoptosis Response Protein 4 (par4) has been identified as a WT1 interacting protein (Johnstone *et al.*, 1996; Richard *et al.*, 2001). The original identification of par4 as a WT1-interacting protein proposed that par4 enhances transcriptional repression by WT1 as shown by transient transfection studies (Johnstone *et al.*, 1996). In addition, this study showed that the zinc fingers of WT1 mediate the interaction between WT1 and par4. Recently it has been

shown that par4 also interacts with the 17 amino acid mammalian-specific alternative splice form of WT1 (Richard *et al.*, 2001). These 17 amino acids are encoded by exon 5 and have been shown to constitute a separate transcriptional activation domain (Richard *et al.*, 2001). The function of the alternative splice activation domain requires a direct interaction with par4. Analysis of this interaction demonstrated that par4 enhances the ability of WT1 to regulate cell survival and proliferation (Richard *et al.*, 2001).

Using the N-terminal domain of WT1 as bait in a yeast two-hybrid screen identified a hUBC9 as an interacting partner (Wang *et al.*, 1996). HUBC9 encodes the human homologue of the yeast ubiquitin-conjugating enzyme 9. In yeast, UBC9 is a nuclear protein involved in the degradation of S- and M-phase cyclins, however the significance of an interaction with WT1 has yet to be ascertained. The association of WT1 and hUCB9 may impose a cell cycle block by affecting the fate of the S- and M-phase cyclins that are degraded by ubiquitination.

6.1.2.3 RNA Metabolism

Involvement of WT1 in RNA splicing and post-transcriptional regulation and has been demonstrated by the association of WT1 with U2AF65 and WTAP. Given the subnuclear localisation of WT1 +KTS isoforms in splicing speckles and the observation that WT1 co-localises and co-immunoprecipitates with splicing factors (Larsson *et al.*, 1995), a yeast two-hybrid screen was employed to investigate the ability of WT1 to interact directly with components of the splicing machinery. From the components tested, only U2AF65 was identified as a potential binding partner (Davies *et al.*, 1998). Extending this work the authors showed that the domain of WT1 that interacts with U2AF65 lay in the zinc finger region, and that the KTS isoforms displayed differing binding affinities to U2AF65 (Davies *et al.*, 1998). Furthermore the +KTS isoforms demonstrated a higher binding affinity to U2AF65.

Using yeast-two hybrid screens, WTAP (WT1-associating protein), a ubiquitously expressed protein, was shown to interact with the C-terminal zinc finger domains of WT1 (Little *et al.*, 2000). The biochemical role of WTAP and the functional significance of its interaction with WT1 are not yet fully elucidated. However WTAP has recently been shown to be a putative mammalian homologue of the protein encoded by the *Drosophila* female-lethal-2-D gene, *fl(2)d*. Genetic studies have indicated *fl(2)d* is involved in regulation of alternative splicing in *Drosophila*. In studying the functional conversation between WTAP and *Drosophila fl(2)d*, *in vitro* assays have demonstrated that WTAP is capable of replacing the function of *fl(2)d* in splicing regulation of *Drosophila*-specific transcripts, and therefore may have a role in splicing (Ortega *et al.*, 2002). Further evidence to implicate WTAP in splicing has been provided by the proteomic analysis of the human splicesome, which identified WTAP as being a member protein (Zhou *et al.*, 2002). The observation of an interaction of WT1 with U2AF65 and WTAP, has strengthened the case for WT1 playing a role in post-transcriptional processing and RNA metabolism by linking it directly to the components of the splicing machinery.

Overall, WT1 is a multi-functional protein that has been implicated in a diverse range of cellular activities including transcriptional control, cell cycle regulation, apoptosis and RNA splicing. The aim of the work presented in this chapter was to isolate and identify novel WT1-interacting proteins. By identifying novel WT1-interacting proteins, a greater understanding of the molecular pathways that WT1 is involved in can be gained.

The majority of studies that have identified interacting WT1 partners have employed co-immunoprecipitation of ectopically expressed WT1 isforms or yeast two-hybrid screens. These approaches have produced a list of potential WT1 interacting proteins (Table 6.1). To date, however there have been no reports of techniques that have utilised immunoprecipitaiton (IP) of endogenous WT1 to identify interacting WT1 proteins. As shown in Chapter 4, *all-trans*

Interacting Proteins	Type of Protein	Subcellular Localisation	WT1 Interacting Domain	Pathway/Function
p53, p63 & p73	Transcription Factors	Nuclear	Zinc Fingers	Cell Cycle Regulation and Transcriptional Regulation of WT1
WTAP	Unknown	Nuclear	Zinc Fingers	Possible role in Splicing
HSP70	Molecular Chaperone	Nuclear Speckles	N-Terminal Domain	Permits WT1 Growth Inhibition
PAR4	Transcription Factor	Nuclear and Cytoplasmic	Exon 5 and Zinc Fingers	Represses WT1 Mediated Transactivation
Ciao 1	WD40 Containing Protein	Nuclear and Cytoplasmic	Zinc Fingers	Represses WT1 Mediated Transactivation
hUBC9	Ubiquitin Conjugating enzyme	Nuclear	N-Terminal Domain	Possible Function in Cell Cycle Progression
U2AF65	Splicing Factor	Nuclear	Zinc Fingers	Role in pre-mRNA Splicing
SF1	Orphan Nuclear Receptor	Nuclear	Zinc Fingers	Activation of MIS/Gonadal Development
BMZF2	Transcription Factor	Nuclear	Zinc Fingers	Repress WT1 mediated transcription

Table 6.1 Published WT1 Interacting Proteins

(Compiled from Reviews by Little *et al.*, 1997 and Lee and Haber, 2001)

retinoic acid (RA) induction of wild-type ES cells (E14) results in high level expression of endogenous WT1. Therefore, the aim of this work was to use this *in vitro* system in conjunction with WT1 IPs and MALDI-TOF to identify endogenous proteins which interact with WT1. The establishment of WT1 null ES cells provides an ideal control for the identification of endogenous WT1 associated proteins. Comparing IPs from the differentiated wild-type ES cells alongside the WT1 null ES cell (K1), permits a control to be included. Simply, anything which is shown to be present in the IPs from the WT1 null ES cells can be discounted as artifactual due to the absence of WT1 in this system.

6.2 Immunoprecipitation of Endogenous WT1 from RA Differentiated ES Cells

To identify WT1-associated protein partners, immunoprecipitation of endogenous WT1 was carried out on nuclear extracts prepared from E14 ES cells treated with RA for 96 hours. As a control for the immunoprecipitation, the K1 ($WT1^{-/-}$) ES cell line was also differentiated with RA for 96 hours, and nuclear extracts prepared. The immunoprecipitations for WT1 were carried out using the C-19 antibody, which recognises the C-terminal region of WT1, bound covalently to protein A-Sepharose beads. Following IP, approximately 10μg of total nuclear protein, 10μg of unbound material and 10μg of elutions from the sepharose beads were separated on a 10% SDS-PAGE gel and transferred onto nitro-cellulose. Western Blot detection for WT1 was carried using the WT1 monoclonal antibody (H2) (Figure 6.1). Western blot clearly shows an enrichment of WT1 in the IPs from the differentiated E14 ES cells, whilst WT1 was not detected in samples immmunoprecipitated from differentiated K1 ES cells.

The WT1 IP samples from E14 and K1 were then separated on a gradient SDS-PAGE gel and stained with Coomassie Blue (Figure 6.2). Five bands were specifically precipitated with C-19 in the wild-type extracts (L1-L5), but not

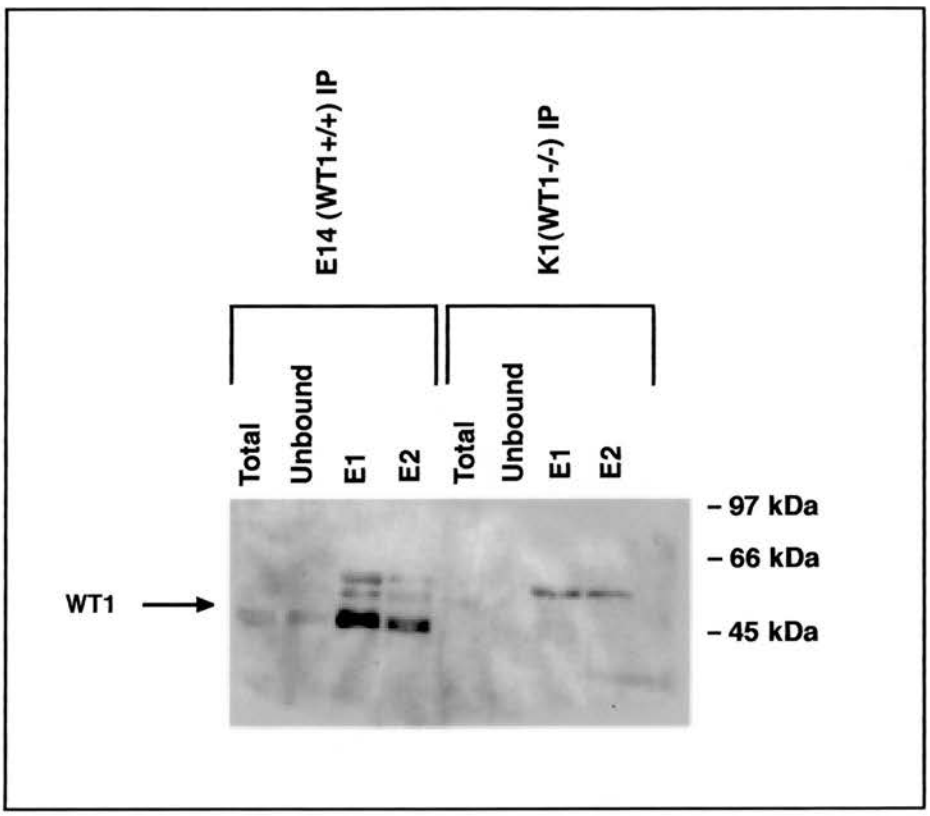


Figure 6.1 The C-19 antibody immunoprecipitates WT1 from wild-type RA differentiated ES cells (E14 IP), but not from RA differentiated WT1 null ES Cells (K1 IP)

ES cells (E14 and K1) were differentiated for 96 hours with 1μM *all-trans* Retinoic Acid and nuclear protein was prepared. The nuclear extracts were immunprecipitated with the C-19 antibody. Approximately 10 μg of T=Total nuclear protein, UN=unbound material, E1/E2=eluate was loaded for Western blotting. Immunoblot for WT1 was carried out using the WT1 mouse monoclonal (H2).

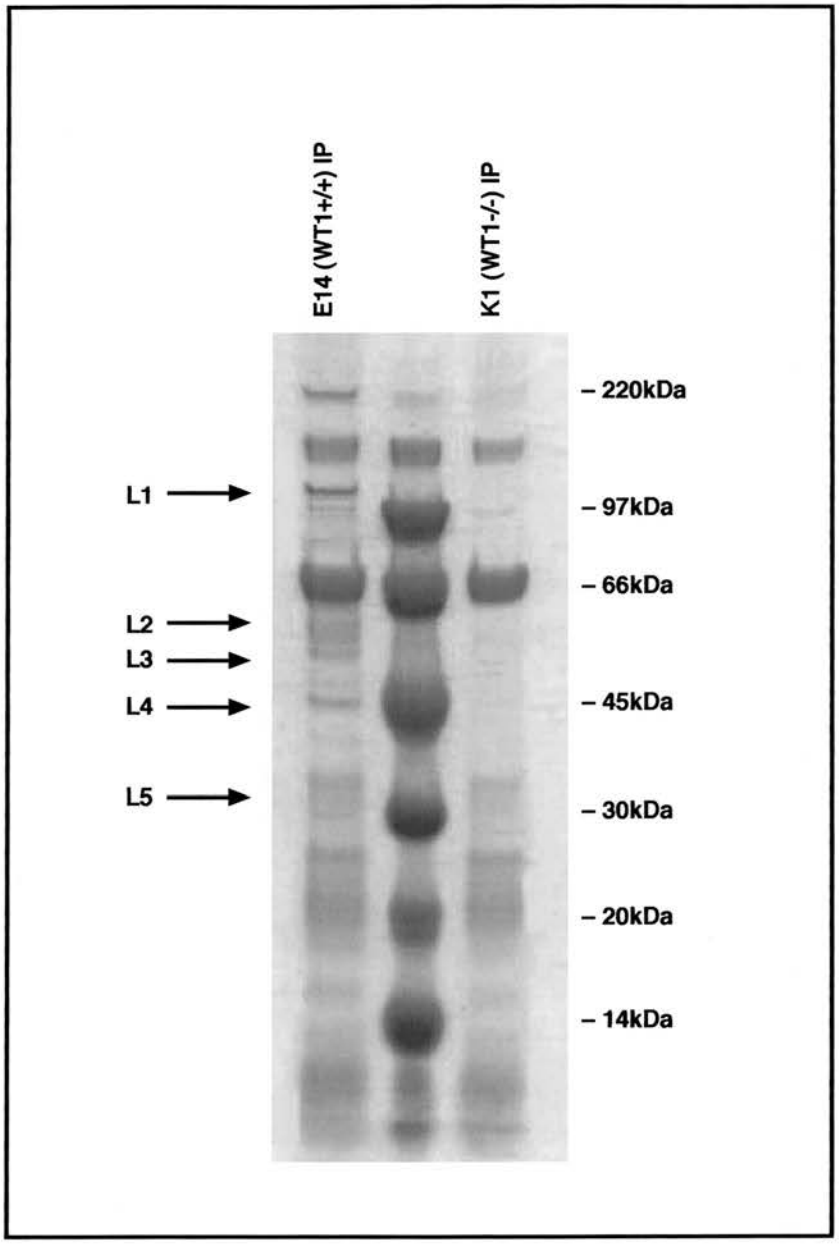


Figure 6.2 Coomassie Blue Stained Gradient SDS-PAGE gel of WT1 IP from E14 and K1 RA Treated ES Cells

20μg of protein from the C19 IP from E14 and K1 was separated on a gradient SDS-PAGE gel and stained with Coomassie blue. Bands that were present in the E14 IP and absent in the K1 IP were labeled L1–L5. These bands were excised from the E14 IP lane for protein identification by MALDI-TOF.

from the K1 null ES cells. These bands were excised from the gel, along with the corresponding regions from the K1 control lane (LN1-LN5).

6.3 Identification of WT1 Interacting Proteins by MALDI-TOF

A tryptic digestion of each of the 5 samples (L1-L5) was performed and the molecular mass of the tryptic fragments determined by MALDI-TOF. The proteins were identified from each band by matching the observed proteolytic masses obtained from the MALDI-TOF spectra with the hypothetical tryptic peptide masses derived from the NCBI non–redundant protein database (MS-Fit) (Table 6.2).

L1 was shown to be highly similar to heterogeneous nuclear ribonucleoprotein U (hnRNP U). Analysis of the LN1 sample from the WT1 null ES cells produced no protein match. Analysis of L4 showed high similarity to either γ or β-actin, whilst L5 showed no protein matches with the NCBI MIS-FIT database. Due to technical problems associated with the analysis of the samples by MALDI-TOF, samples L2 and L3 could not be processed or identified.

6.4 Heterogeneous Nuclear Ribonucleoprotein U (hnRNP U)

From the MALDI-TOF analysis, the protein identified from the L1 band, hnRNP U, was chosen for further investigation. This band was the most prominent in the Coomassie-stained SDS-PAGE gel of the E14 WT1 IP. HnRNP U (also known as Scaffold Attachment Factor A, SAF-A) is a 120 kDa protein belonging to the heterogeneous nuclear ribonucleoproteins family of proteins (hnRNPs) that are abundant in the cell nucleus (Kiledjian and Dreyfuss, 1992).

Sample	Molecular Mass	Number of Matching Peptides	Protein Identification	NCBI Accession Number
L1	> 97 kDa	17	hnRNP U	gi:14044052
L2	45 kDa – 60 kDa	—	—	—
L3	45 kDa – 60 kDa	—	—	—
L4	45 kDa	12	γ/α-actin	gi: 809561
L5	30 kDa – 45 kDa	No Match	No Match	No Match

Table 6.2 Analysis of L1-L5 Bands by MALDI-TOF

Samples L1-L5 were excised from the Coomassie stained gradient gel and analysed by MALDI-TOF. Interpretation of the data generated from the MALDI-TOF was carried out using the MS-Fit program.

6.4.1 WT1 and HnRNP U Interact *in vivo*

To ensure that hnRNP U is present in cell types that have arisen from the WT1 null ES cells following exposure to RA, nuclear protein from RA differentiated wild-type E14 ES cells and K1 ES cells were probed for hnRNP U using 3G6, a mouse monoclonal antibody against hnRNP U (Gift Dr. Gideon Dreyfuss). Nuclear protein (40 μg) was separated on a 10% SDS-PAGE gel, transferred to nitrocellulose and probed with the 3G6 antibody. Figure 6.3 (b) shows the Western blot for hnRNP U. In both E14 and K1 nuclear protein, a single band migrating above 97 kDa can be detected. This is in comparison to the Western blot for WT1 which demonstrates its presence only in the RA induced E14 ES cells (Figure 6.3 (a)). The blot was then stripped and reprobed with an antibody for P116 to ensure equal loading of nuclear protein (6.3 (c)). The Western blot analysis shows that the absence of a band corresponding to L1 in the K1 WT1 null ES cells is not due to an absence of hnRNP U in the original sample.

To confirm that hnRNP U interacts with WT1, an independent set of IP experiments were carried out on further nuclear protein extracts collected from wild-type ES cells differentiated for 96 hours with RA. IPs for WT1 (using C-19), hnRNP U (using the mouse monoclonal antibody 3G6), and a negative control IP (using rabbit IgG), were carried out. Proteins (10μg) from the elutions of the IPs (E1 and E2) were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose. Figure 6.4 shows the Western blot analysis of the different IPs.

Figure 6.4 (a) is a Western blot of the C-19 WT1 IP elutions probed with both H2 (α WT1) and 3G6 (α hnRNP U). It shows that a doublet migrating between 45 kDa and 60 kDa was detected corresponding to WT1, and also a single band migrating above 97 kDa, which represents hnRNP U. The presence of both

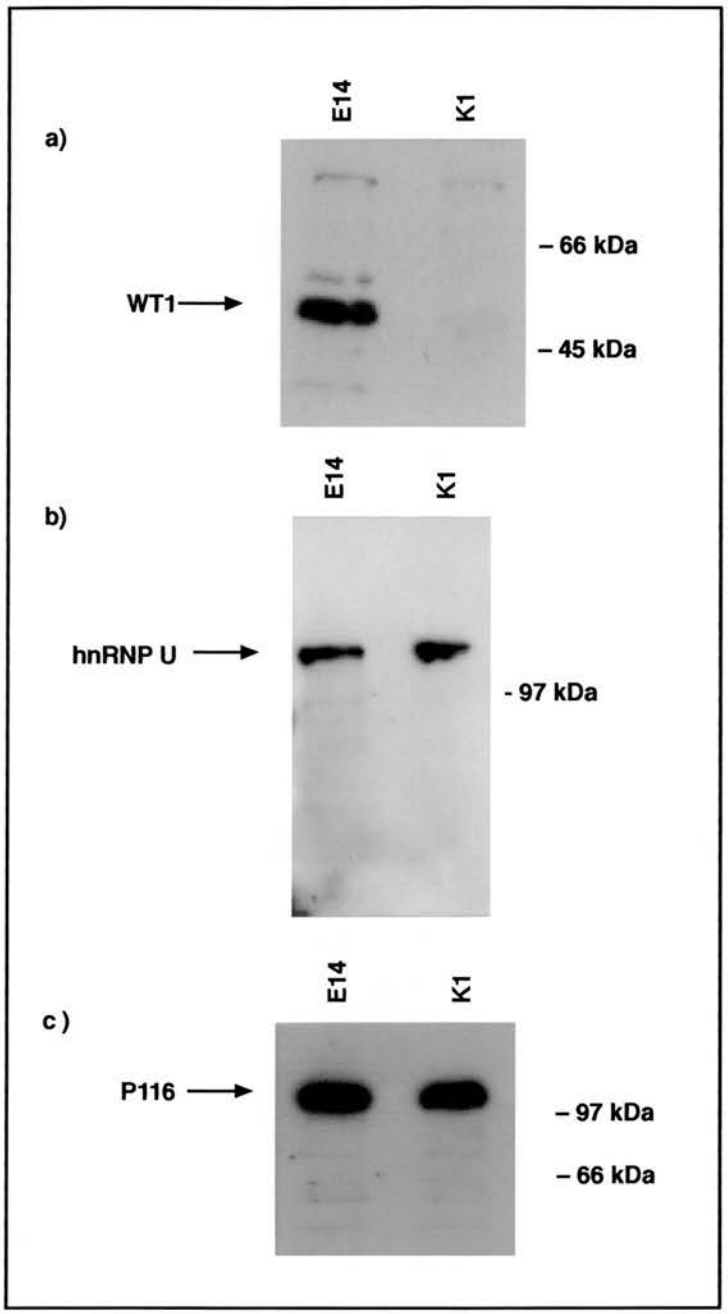


Figure 6.3 HnRNP U is Expressed in Both RA Treated E14 and K1 ES Cells After 96 Hours

ES cells (E14 and K1) were treated with RA for 96 hours and nuclear protein was prepared. Immunoblot of the nuclear protein (40 μg) for WT1, HnRNP U and P116 was carried out.

a) Both samples were probed for WT1 using the C-19 antibody, demonstrating an absence of WT1 in the K1 clone.

b) Both samples were probed for hnRNP U using 3G6 antibody, demonstrating that hnRNP U is present in both clones.

c) To control for loading, the blot was stripped and probed with an antibody to P116, a U5 small nuclear RNP-associated protein.

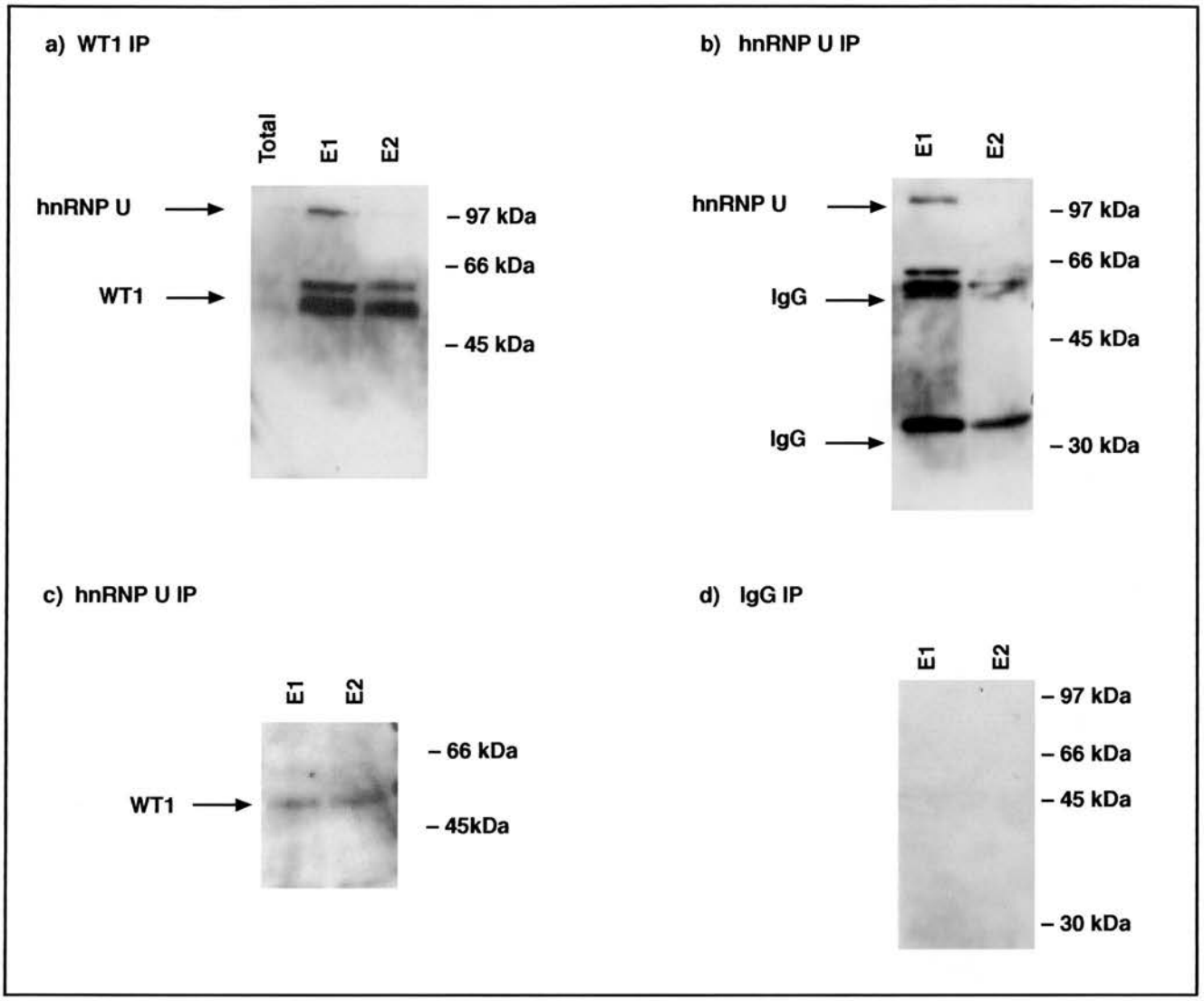


Figure 6.4 WT1 interacts with hnRNP U *in vivo*

E14 ES cells were treated with RA for 96 hours and nuclear protein extracts prepared.

a) Immunoprecipitations for WT1 were carried out using C-19. 10 μg of protein from the IP was separated on a SDS PAGE gel and transferred onto nitrocellulose. The immunoblot was probed with the monoclonal antibodies for WT1 (H2) and hnRNP U (3G6). WT1 and hnRNP U are both present in the elutions from the WT1 IP.

b) Immunoprecipitations for hnRNP U were carried out using 3G6. The immunoblot was probed with the 3G6 antibody to ensure that hnRNP U can be immunprecipitated. HnRNP U can be detected in the elutions.

c) Immunoblot from (b) was stripped and reprobed for WT1 using the rabbit polyclonal C-19. WT1 can be detected in the elutions (E1 and E2) from the hnRNP U IP.

d) Control immunoprecipitations using a rabbit IgG were carried out on nuclear extract from RA treated E14 ES cells. The elutions were probed with for both WT1(H2) and hnRNP U (3G6). There was no detection of hnRNP U or WT1.

WT1 and hnRNP U in the WT1 IP elution E1 indicates that they physically interact in so far as they co-precipitate together.

The hnRNP U IP using the 3G6 antibody, was then probed again with the same antibody to ensure that hnRNP is immunoprecipitated from the nuclear protein. In the elutions from the hnRNP U IP, hnRNP U has been enriched in comparison to the total nuclear protein (Figure 6.4 (b)). The bands at approximately 66 kDa and 30 kDa are non specific and correspond to the product of cross reactivity from the anti-mouse secondary HRP antibody used in the Western blot detection, which interacts with the mouse IgG chains from the mouse monoclonal hnRNP U antibody (3G6).

The blot for the hnRNP U IP was then stripped and reprobed with the WT1 C-19 antibody (Figure 6.4 (c)). In the hnRNP U IPs, a band above 45 kDa corresponding to WT1 can be detected in the IP (Figure 6.4 (c)). Western blotting of the IgG IP with both H2 and 3G6 showed no detectable bands for either WT1 or hnRNPU (Figure 6.4 (d)).

6.5 Discussion

Utilising endogenous immunoprecipitations from differentiated ES cells, coupled to MALDI-TOF analysis, we have identified an interaction of WT1 with hnRNP U. The precise role of hnRNP U *in vivo* is not fully understood, but evidence suggests that it is most likely involved in at least three different processes. It has been demonstrated that hnRNP U acts as a transcriptional repressor (Kim and Nikodem, 1999). Secondly, as a constituent of the hnRNP particles, hnRNP U is involved in the packaging and processing of RNA (Dreyfuss *et al.*, 1993). And, finally, several reports have indicated that hnRNP U has a high binding-specificity for specialised AT-rich DNA regions termed SARs (Scaffold-Associated Regions). Each of the proposed functions for hnRNP U will be discussed in the context of the association of WT1 with hnRNP U.

6.5.1 Transcriptional Repression

A recent study has identified hnRNP U as an RNA polymerase II elongation inhibitor (Kim and Nikodem, 1999). Eukaryotic mRNA synthesis is catalysed by the multi-subunit RNA polymerase II (RNA Pol II) and proceeds through multiple steps, designated pre-initiation, initiation and elongation. The RNA Pol II transcription complex possesses a unique structural feature, the carboxyl terminal domain (CTD). The phopshorylation state of this CTD domain regulates the transcriptional activity of RNA Pol II. In an unphosphorylated state, the RNA Pol II CTD is believed to mediate multiple protein-protein interactions involved in the assembly of the pre-initiation complex. However, once initiation of transcription begins, kinases phosphorylate the CTD domain to generate the elongation-competent RNA Pol II and the generation of a primary transcript (For review see Bentley, 1998). One of the kinases that is believed to phosphorylate the CTD is the multi-subunit general transcription factor, TFIIH.

It has been demonstrated that a fraction of hnRNP U is found associated with the RNA Pol II complex and is recruited to a promoter as part of the pre-initiation complex (Kim and Nikodem, 1999). HnRNP U then dissociates from the RNA Pol II complex at an early stage of transcription and is therefore absent from the elongation competent form of RNA Pol II. The authors were able to demonstrated that hnRNP U acts as an elongation inhibitor by inhibiting the kinase activity of TFIIH, thereby preventing the phosphorylation of the CTD and acting as a transcriptional repressor (Kim and Nikodem, 1999). How does this fit into a situation regarding the known function of WT1?

Transcriptional regulation by WT1 appears complex, with a number of different factors know to influence whether WT1 acts as a repressor or activator of transcription. Dissection of the WT1 transcriptional regulation domains using GAL-4 fusion proteins has identified distinct domains responsible for either

transcriptional activation or repression (McKay *et al.*, 1999; Wang *et al.*, 1995; Madden *et al.*, 1993). Studies have indicated that a transcriptional repressor domain is located between amino acids 70-180 in the N-terminal domain of WT1 (Wang *et al.*, 1995; Madden *et al.*, 1993). More recent studies have further refined the repressor domain to amino acids 71-101 (McKay *et al.*, 1999). This 30 amino acid domain was demonstrated to function as a transcriptional repressor in a WT1-independent context by fusing this domain to the activation domain of SP1 (McKay *et al.*, 1999). However, questions still remain as to how the WT1 transcriptional repressor domain exert its effects and the level at which WT1 induces transcriptional repression in the context of the molecular pathway of RNA Pol II.

Both studies by Wang *et al* and McKay *et al*, have suggested that this WT1 repressor domain associates with another nuclear protein which in turn mediates the effects of WT1 as a transcriptional repressor (Wang *et al.*, 1995; McKay *et al.*, 1999). With regard to the issue of at which point WT1 represses transcription, a study by Lee *et al* has shown that the same WT1 domain is capable of repressing co-activators that stimulate the initiation and/or elongation steps of RNA Pol II (Lee *et al.*, 1999). The authors suggested that a possible mechanism by which WT1 could achieve this repression of co-activators is by a direct interference with the basal transcriptional complex. Therefore it could be suggested that the association of WT1 with hnRNP U facilitates the transcriptional repression elicited by WT1. This would seem feasible, as there has yet been no description of a direct association of WT1 with the components of the RNA Pol II complex. Therefore, hnRNP U may provide a bridging partner via which WT1 may mediate some aspects of transcriptional regulation.

6.5.2 RNA Processing

Whilst the elongating RNA Pol II complex is generating a primary transcript, the messenger RNA processing reactions of capping, splicing and polyadenylation are occurring co-transcriptionally. The hnRNP family of

proteins are RNA processing factors that associate with the pre-mRNAs as it emerges from the RNA Pol II, acting to facilitate the various stages of mRNA biogenesis (For review see Dreyfuss *et al.*, 2002). This interaction with RNA is mediated through the presence of an RNA- binding domain. Most, if not all, of the hnRNP proteins contain one or more of a small number of RNA-binding motifs. HnRNP U contains a RGG domain that mediates its association to RNA, and also to other hnRNP proteins to form hnRNP particles. Given the reported association of WT1 with U2AF65 and WTAP, both components of the splicing machinery, an association with hnRNP U might be another protein partner that facilitates the RNA-processing function of WT1 (Davies *et al.*, 1998; Little *et al.*, 2000). This association might be governed, as with U2AF65, through the zinc fingers of WT1, and in particular the +KTS isoforms. Added to this is the fact that WT1 has a putative RRM domain and also the zinc fingers of WT1 have been shown to bind RNA (Kennedy *et al.*, 1995; Caricasole *et al.*, 1996).

6.5.3 Scaffold Attachment Regions

Within the eukaryotic nucleus, chromosomal DNA is organised into higher order structures called chromatin which itself is organised within the nucleus. These chromatin structures are established by attaching the chromosomal DNA to proteins of the nuclear matrix, a proteinaceous structure that serves as a scaffold. This association is mediated by DNA regions termed Scaffold Attachment Regions (or SARS), that anchor the chromosomal DNA to the nuclear matrix. In addition to their role in organising nuclear architecture, SARs have also been implicated in the regulation of gene expression. This has been proposed due to a number of observations. Firstly they are frequently found in genomic locations close to enhancers (Cockerill *et al.*, 1986) Secondly, they can also stimulate gene expression of heterologous reporter gene when integrated into the genome (Stief *et al.*, 1989), and thirdly, they can regulate chromatin accessibility (Jenuwein *et al.*, 1997). Several SAR binding proteins have be characterised including hnRNP U (otherwise know as Scaffold Attachment Factor A, SAF-A). Apart from possessing an RNA-binding domain, hnRNP U

also contains a novel DNA-binding domain which specifically associates to the scaffold-associated region (SAR) (Gohring *et al.*, 1997). This domain is independent of the RNA-binding domain.

In some cases the activation of gene expression is thought to be dependent on processes involving changes to the chromatin structure (Tolhuis *et al.*, 2002). Therefore, in the context of WT1 an association of WT1 with hnRNP U might lead to alterations in chromatin structure, hence leading to transcriptional activation or repression by WT1. It is interesting to note that WT1 has been observed to exert repression over a large distance during *in vitro* transcriptional assays (Lee *et al.*, 1999). Therefore, an association with hnRNP U might facilitate this effect by modulating chromatin structure.

In summary, the association of WT1 with hnRNP U, a multifunctional protein, may mediate all facets of WT1 function at both the transcriptional and post-transcriptional level.

Chapter 7 General Discussion and Future Directions

Chapter 7 General Discussion and Future Directions

7.1 Generation of Genetic Model Systems to Investigate the Role of WT1

The *WT1* gene was identified as a tumour suppressor gene involved in the etiology of Wilms' tumour. To date, it is clear that the biology of WT1 is complex. Studies have indicated that in addition to its function as a tumour suppressor, this gene has multiple roles during development. This is exemplified in the WT1 mouse models, which have demonstrated the critical importance of WT1 expression for the development of several organs, including the kidneys, gonads, spleen and, more recently, the retina. This complexity of WT1 action during development is also reflected at the molecular level. Alternative splicing and the use of alternative translation initiation sites, generate a multitude of isoforms which seem to have overlapping, but also distinct, functions during embryonic development. The WT1 proteins seem to perform two main functions. They regulate the transcription of a variety of target genes and may be involved in post-transcriptional processing of RNA. Over the last few years our understanding of WT1 function has significantly improved, however there are still many unanswered questions. What are the transcriptional targets of WT1? What is the function of WT1 in the later stages of embryogenesis and post-natally? Therefore, the aim of this thesis was to develop new genetic models to begin to address some of these questions, and gain further insight into *WT1* gene function.

7.1.1 Generation of a Conditional Allele for WT1

The first part of this project was to develop a conditional allele for *WT1* utilising the *Cre/LoxP* system. This strategy would bypass the embryonic lethality of the original WT1 knockout, and enable the role of WT1 during embryogenesis and

post-natal development to be further investigated (Kreidberg *et al.*, 1993). Initially, the generation of mice carrying the germline modifications of the *WT1* locus was problematic. The likely cause of this was a *Cre*-mediated genomic rearrangement at the 3' end of the targeted *WT1* allele, which occurred during the transient expression of *Cre*-recombinase. Analysis of an ES cell line carrying the targeted allele following *Cre* recombination demonstrated that the intronic region where the 3' *loxP* site resided had undergone genomic rearrangement. Therefore, another independent ES cell line was treated with *Cre* and carefully analysed. Individual clones selected from this transfection did not demonstrate any aberrant rearrangements in the 3' region, and ES clones with *loxP* sites flanking exon 1 of *WT1* were successfully used to obtain germline transmission of the *WT1* conditional allele. To ensure that the modifications to the *WT1* allele did not produce a phenotype, heterozygote *WT1* conditional mice were mated. Normal-sized litters were obtained with the expected Mendelian frequency of wild-type, heterozygote and homozygote genotypes and homozygous *WT1* conditional mice were phenotypically normal after 12 weeks.

The conditional *WT1* mouse line can now be employed to address some of the questions surrounding the function of WT1 in development and disease. One of the first questions that could be addressed would be the consequence of ablating WT1 during the early stages of metanephric kidney development. Could this induce the onset of a Wilms' tumour? Wilms' tumours are thought to arise from the pluripotent metanephric blastemal cells following the induction of differentiation of the metanephric mesenchyme by the ureteric bud. Ideally, one would like to ablate WT1 following induction of the metanephric mesenchyme. At present, there are no suitable transgenic *Cre* lines available to conditionally inactivate WT1 during this stage of kidney development. This makes it difficult to examine the role of WT1 during this inductive phase of nephrogenesis, and whether loss of WT1 function would lead to a predisposition to Wilms' tumours in this model. To overcome this

difficulty, the conditional *WT1* mouse would be crossed onto transgenic mice that carry an inducible form of *Cre*-recombinase under the control of the human WT1 YAC. These transgenic lines are currently in this laboratory (Hastie *et al.*, unpublished). This human WT1 YAC has been show to direct beta-galactosidase expression in a manner that recapitulated the endogenous WT1 expression pattern (Moore *et al.*, 1998; Moore *et al.*, 1999). Therefore, this approach might be suitable to ablate WT1 during this phase of kidney development.

What is the consequence of knocking out WT1 in the adult podocyte? Although WT1 expression has been reported in a variety of tissues during development, one site of WT1 expression that is maintained into adulthood is in the glomerular podocytes of the kidney. Recent observations indicate that WT1 expression in this cell-type is required for the maintenance of normal kidney function in adulthood (Guo *et al.*, 2002; Menke *et al.*, in press). The analysis of heterozygote WT1 mice demonstrates that they exhibit an increased mortality rate due to adult onset renal failure. Whether WT1 expression is crucial to podocyte function can now be directly tested by crossing the conditional *WT1* mouse onto the recently published transgenic mouse line carrying a glomerular-specific inducible *Cre*-recombinase (Bugeon *et al.*, 2003).

In summary, the successful generation of a conditional *WT1* mouse leaves us well-placed to address the numerous questions relating to the role of WT1 in development and disease, which is limited only by the availability of suitable transgenic *Cre* lines.

7.1.2 Generation of WT1 Null ES Cell Lines

The second part of this approach was to generate WT1 null ES cell lines. These ES cell lines would then be used in an *in vitro* differentiation system to identify

candidate WT1 regulated transcripts. In generating the WT1 null ES cells, the original strategy was to engineer ES cells that carried a conditional allele of *WT1*. In this system, the loss-of-function for WT1 would be under the spatial-temporal control of *Cre*-recombinase. However targeting of the second wild-type *WT1* allele proved difficult, as several attempts failed to correctly target the *WT1* allele and incorporate the three *loxP* sites. Therefore, the original targeting vector used in the generation of the WT1 knockout mouse (Kreidberg *et al.*, 1993) was used to target the second remaining *WT1* to produce two independent lines of WT1 null ES cells.

7.2 Identification of Candidate WT1 Regulated Transcripts

A number of approaches have been employed over the last decade in an attempt to elucidate the network of genes that are regulated by WT1. The majority of published downstream targets of WT1 have been identified from experimental approaches that have employed either the over-expression of single WT1 isoforms followed by analysis of gene expression profiles, or transient transfections with engineered reporter constructs. The results from these experiments have produced a list of putative WT1 target genes which includes other transcription factors, growth factors, growth factor receptors, extracellular matrix genes, cell survival genes and signaling molecules amongst others (For review see Lee and Haber, 2001). However, such studies suffer from several disadvantages. From the over-expression of WT1 isoforms and subsequent analysis of downstream gene expression profiles, differentially expressed genes identified by this process may not necessarily be direct targets of WT1. Rather, it is possible that the differential expression of a gene is due solely to indirect effects of over-expression of WT1. More importantly, it is unclear whether the genes influenced from over-expression of WT1 represent true *in vivo* targets of WT1, where WT1 is present at physiologically relevant concentrations. Also, the over-expression studies have focused on single isoforms

of WT1, whereas it is clear that WT1 isoforms and their relative ratios are crucial for normal physiology. Similarly the results from the transient transfections of reporter constructs vary significantly depending upon experimental parameters such as the choice of cell line and expression vectors, the WT1 isoform expressed and the promoter architecture of the putative target (Reddy and Licht, 1996). More importantly, often the *in vivo* relevance of the previously reported candidate downstream WT1 target gene remains unclear.

In an attempt to address some of these issues, it was critical to develop a system in which endogenous targets of WT1 could be identified under biologically relevant conditions. A previous report demonstrated that the differentiation of embryonic stem cells with *all-trans* retinoic acid (RA) as a monolayer culture induced high level endogenous expression of WT1 (Scharnhorst *et al.*, 1997). Hence this system provided an ideal platform with which to study WT1 function in a pluripotent, non-transformed cell line which retains the potential to differentiate into multiple lineages. Therefore the approach taken in this thesis was to develop WT1 null ES cell lines (described above) and to use these in a RA differentiation system. Coupled to Affymetrix Genechip arrays, this would permit the identification of candidate WT1 regulated genes, which were affected by loss-of-function for WT1 following differentiation.

Of the 12,000 transcripts present on the array, only a small number (102) showed a difference of greater than 2.5-fold change in expression between wild-type and WT1 null ES cells. The list of differentially expressed transcripts included genes that had previously been identified as being regulated by WT1, including amphiregulin, Wnt-4 and the murine homologue of FREAC-4, BF-2 (Lee *et al.*, 1999; Sim *et al.*, 2002; Ernstsson *et al.*, 1996). However, the remaining transcripts represent a novel set of candidate WT1 regulated genes.

An important caveat to this study is that the data sets generated from the initial Affymetrix analysis of wild-type and WT1 null ES cell gene expression profiles is preliminary. Whilst providing some very interesting candidate WT1 targets, this study requires further replicate samples to be analysed by microarray, to provide a comprehensive statistical analysis. However differential expression of a number of those putative targets was confirmed by RT-PCR analysis. Following on from this, it is vital to determine whether these differentially expressed transcripts are directly regulated by WT1. Various co-transfection approaches could be employed to determine this. However Chromatin immunoprecipitation (ChIP) analysis is a useful technique which detects a direct *in vivo* interaction between any transcription factor and its binding site on genomic DNA. This approach has been successful in identifying transcripts which are regulated by other transcription factors, including E2F (Weinmann *et al.*, 2002) and c-Myc (Fernandez *et al.*, 2003).

Briefly this experimental approach would require wild-type and WT1 null ES cells to be treated with RA, followed by formaldehyde cross-linking, chromatin isolation and DNA shearing by sonication. Immunoprecipitation would then proceed using the WT1 antibody to selectively precipitate WT1 protein and any DNA fragments cross-linked to it. Thus, DNA sequence elements associated with WT1, in the context of the cellular environment, would be enriched in the immunoprecipitated sample. A PCR screen using primers specific for promoters of candidate WT1 target genes would indicate whether it had been bound by WT1.

In summary, the identification of a novel set of candidate WT1 regulated genes may lead to the elucidation to the mechanism by which WT1 mediates its downstream effects *in vivo*.

7.3 Neuronal Expression of WT1

During embryogenesis, WT1 is expressed in a tissue specific manner. Studies have identified sites of expression in the urogenital system, the epicardium of the heart, the spleen, and mesothelium (Armstrong *et al.*, 1993; Moore *et al.*, 1998; Moore *et al.*, 1999). Consistent with this expression pattern is the phenotype of the WT1 knockout mouse, which displays gonadal and renal agenesis in addition to heart, spleen and adrenal abnormalities. To date, the role of WT1 in kidney development has received the most attention. However the reported expression of WT1 in neuronal lineages is yet to be further characterised. Initial reports have described discrete pockets of expression in the developing eye, spinal cord and brain (Armstrong *et al.*, 1993; Rackely *et al.*, 1993; Moore *et al.*, 1998). A recent study has indicated that WT1 expression in the eye has a role in the development of the retina. Analysis of WT1 knockout mice has revealed abnormalities in retinal development, with optic nerve hypoplasia and apoptotic loss of retinal ganglia cell precursors (Wagner *et al.*, 2002). The data presented in this thesis extends the analysis of WT1 expression in neuronal lineages.

In the analysis of the differentiating ES cells, it was observed that a sub-population of WT1 positive cells co-expressed nestin. This indicated that WT1 was being induced in a subpopulation of neuronal progenitor cells. To confirm this finding, the expression of WT1 in neuronal progenitors that were derived from the differentiation of a genetically modified ES cell line was analysed (Li *et al.*, 1998). This *in vitro* lineage selection protocol enables the efficient purification of neuroepithelial progenitor cells, which subsequently differentiate efficiently into neuronal networks at the expense of other cell types. Semi-quantitative RT-PCR demonstrated the up-regulation of WT1 expression during the initial stages of neuronal progenitor cell differentiation.

Next, the analysis of WT1 expression in neuronal lineages was extended *in vivo*. Interestingly expression of WT1 appeared to be present in a domain where neuronal progenitors are generated. Focusing on E9.5-E10.5, it was evident at E10.5 that WT1 was expressed in the ventral neural tube. Closer examination indicated that in the developing ventral neural tube, WT1 expression was present in domains that contained neuronal progenitors, as demonstrated by co-localisation of WT1 with Nkx6.1. However, it was unclear exactly which specific neuronal progenitor subtype expressed WT1. In the ventral neural tube, the combinatorial expression of a group of homeodomain proteins generates five different neural progenitor subtypes (For review see Briscoe and Ericson, 2000). Nkx6.1 marks the three most ventral progenitors subtypes (p3, pMN, p2). To specifically identify which domain expresses WT1, double immunofluorosence using specific markers for the p3, pMN and p2 domain alongside WT1 would need to be carried out. However is was clear that WT1 was expressed in differentiated motor neurons, as demonstrated by co-localisation of WT1 with the Lim homeodomain protein, Islet-1. Therefore, this data indicates that WT1 is expressed in certain neuronal progenitor cell populations and also in differentiated motor neuron populations of the developing spinal cord.

To further characterise this the analysis of WT1 expression will be extended to later stages of neuronal development, beyond E10.5. This would indicate whether WT1 expression persists in these populations of motor neurons, or whether it is subsequently down-regulated. Does expression in the motor neurons persist into adulthood? What is the function of WT1 in the developing neural tube? Does WT1 have a role in the maintenance of neuronal progenitors, or is it involved in the differentiation of these progenitors towards motor neuron lineage? The two systems described in this thesis will allow further examination, both *in vitro* and *in vivo*, of the function of WT1 in these neuronal lineages. Recently, it has been demonstrated that embryonic stem cells can be directed to differentiate into motor neurons under the correct inductive signals (Wichterle *et al.*, 2002). During this differentiation

process, the ES cell derived motor neurons follow a developmental pathway that recapitulates the situation *in vivo*. It would be interesting to utilise the both wild-type and WT1 null ES cells in this differentiation system to examine if WT1 has a fundamental role in motor neuron generation.

Complementary to the *in vitro* work, the conditional *WT1* mouse will prove invaluable in dissecting whether the specific ablation of WT1 expression in the motor neuron populations results in a phenotype. If future co-localisation studies indicates that WT1 is expressed in motor neuron progenitors, then the use of a *Cre*-recombinase under the control of the Olig2 promoter (Takebayashi *et al.*, 2002) will allow the ablation of WT1 in these specific progenitors. To ablate WT1 expression specifically within the motor neurons, a transgenic mouse line with *Cre* recombinase under the control of Lim3 could be employed (Sharma *et al.*, 1998).

Given that this study has identified WT1 as being expressed in the ventral neural tube, future work will address the target genes that WT1 regulates in the neural tube. The Affymetrix Genechip analysis of gene expression profiles of wild-type and WT1 null ES cells during RA mediated differentiation identified RALDH2 as a differentially expressed transcript. Whether WT1 directly regulates RALDH2 is yet to be established, however it is interesting to note that RALDH2 plays a crucial role in the generation of certain types of motor neuron in the developing spinal cord (Sockanathan and Jessell, 1998). A potential regulation of RALDH2 by WT1 has been already been suggested by studies from co-workers (Professor Munoz-Chapuli, unpublished data). Work focusing on the epicardium of the heart has shown that WT1 and RALDH2 are co-expressed in the same populations of epicardially derived cells. Closer inspection of the WT1 null mice has indicated that RALDH2 expression is down-regulated these cell populations within the epicardium (Professor Munoz-Chapuli, personal communication). This effect is consistent with the observation that RALDH2 expression is down-regulated in

WT1 null ES cells. If RALDH2 represents an authentic target for WT1, the down-regulation of RALDH2 should be a generic feature in the WT1 knockout mice resulting in the down-regulation of RALDH2 in the neural tube.

During spinal cord development, motor neurons with common targets of innervation are clustered into discrete nuclei, termed motor pools. In the neural tube, RALDH2 plays a crucial role in the specification of the lateral motor neuron cell type (LMC) (Sockanathan and Jessell, 1998). This class of motor neurons is generated selectively at branchial and lumbar levels of the spinal cord, and their axons innervate target muscles in the limb. Preliminary analysis of branchial sections from the neural tube of E11.5 WT1 null embryos indicates that RALDH2 expression is in fact down-regulated (Professor Munoz-Chapuli, personal communication). This observation would suggest one of two possibilities: that WT1 is a direct activator of RALDH2 or specification of LMCs has been abolished in the WT1 null, which leads to the observed down-regulation of RALDH2. To address this issue, WT1 null embryos would be analysed with other LMC markers to test for loss of LMC specification. Also, since down-regulation of RALDH2 expression has been shown to effect the number of motor neurons generated (Sockanathan and Jessell, 1998), staining for Islet-1 on WT1 null embryos would address whether the numbers of motor neurons generated has been reduced at the branchial and lumbar regions.

In summary, this study describes the expression of WT1 in neuronal progenitors (*in vitro* and *in vivo*) and also differentiated motor neurons of the developing spinal cord. The demonstration of WT1 expression in both neuronal progenitors and differentiated neurons supports the notion of a role for WT1 in neuronal differentiation. The failure to detect gross abnormalities within the CNS of WT1 null embryos suggests this role may be a subtle one, and closer analysis of these animals is now required to substantiate the initial findings described herein.

7.4 Identification of WT1 Interacting Proteins

The biological function of WT1 *in vivo* is affected by interactions with other proteins. A number of protein partners are known to associate with WT1 and these include p53, SF-1, Par-4, U2AF65 and WTAP (For Review see Lee and Haber, 2001). The interaction of these proteins with WT1 has been shown to modify the transcriptional properties of WT1 and also implicate WT1 in post-transcriptional regulation. Previously, identification of WT1 interacting proteins was based around yeast two hybrid screens, or over-expression of WT1-GST fusions followed by pull-downs to find WT1 protein partners. The approach taken in this thesis was to carry out endogenous immunoprecipitations for WT1 from RA treated ES cells, and then couple this to MALDI-ToF analysis. The data obtained from this strategy identified an interaction of WT1 with a member of the heterogeneous ribonuclear protein family, hnRNP U. Confirmation of this association was achieved by carrying out reciprocal IPs for hnRNP U and WT1, and demonstrating that both co-precipitate together.

The precise function of hnRNP U is unclear. It is known that hnRNP U is involved in transcriptional repression, RNA splicing and possibly, chromatin remodeling. The functional significance of the interaction of WT1 with hnRNP U should now be examined. The first approach will be to identify which domains of WT1 are interacting with hnRNP U. Is their interaction mediated through the zinc fingers of WT1? To determine this, yeast two hybrid studies would be carried out. Is the interaction mediated via common set of RNA transcripts? Does the interaction of WT1 with hnRNP U modulate the transcriptional behaviour of WT1? In summary there are numerous questions relating to the functional significance of the interaction of WT1 with hnRNP U, however it is clear that endogenous WT1 IPs, coupled to mass spectrometry, is a powerful approach to identify WT1 interacting

proteins from complex samples. Future work could extend this approach to identify additional WT1 interacting proteins.

7.5 Conclusion

Following over a decade of study on the biology of WT1, it is clear that this gene is a multifunctional protein that has a complex role in normal development. Whilst the WT1 knockout mice represented an enormous step forward towards the elucidation of these roles, their significance was constrained due to the midgestation lethality observed in this model. The establishment of the two genetic systems described in this thesis will therefore increase the understanding of the functional properties of WT1 and its downstream targets, thereby providing unique insights into the link between normal organ-specific differentiation and malignancy.

Appendix and References

Appendix 1(a) Primer Sequences for PCR

Primer	Sequence
5' LoxP1	GGGGCTTATCTCCTCCCATG
5' LoxP2	CAGTAAGTGTCATCTACACTG
5' LoxP1S	AGAATTCTAGAGGCTAGCTGCGG
5' LoxP2S	TGGGTTCCAACCGTACCAAAG
3' LoxP1	GTGGTTTCGGTTTCGCTAGG
3' LoxP2	GGAAATTCTTTACTGACACAGAC
WT1.1 (PGK)	CTACCGGTGGATGTGGAATGTGT
WT1.2	TCCCGAACAATTTCACCTTGAATC
WT1.3	AGCCTAACTTTGGGGCTTATCTCC

Appendix 1(b) Sequence of Primers used in RT-PCR analysis

Transcript	Sequence 5'-3'
Alpha Fetoprotein	GCTCACACCAAAGCGTCAAC
	CCTGTCAACTCTGGTATCAG
β-Actin	GGCCCAGAGCAAGAGAGGTATCC
	ACGCACGATTTCCCTCTCAGC
βH1	AGTCCCCATGGAGTCAAAGA
	CTGAAGGAGACCTTTGCTCA
BF-2/FoxD1	TGTCCAGTGTGGAGAACTTTACTG
	CTCTACACCTCAAAAAGGGCTTAG
GAPDH	CCCCAATGTGTCCGTCGTGGATCTG
	GATGGTATTCAAGAGAGTAGGGAGGG
Indian Hedgehog	AAGGCCCACGTGCATTGCTCT
	GTCCGCAATGAAGAGCAGGTG
Myf5	TCTAGCTGTTCAGATGGC
	TTAAGAGAGGCTTATAACAC
MyoD	GGAGGCTCTGCTGCGCGACC
	TGCAGTCGATCTCTCAAAGCACC
Nestin	TCCAGAAAGCCAAGAGAAGC
	GGAGTGTCGCTTAGAGGTGC
Oct-4	GGCGTTCTCTTTGGAAAGGTGTTC
	CTCGAACCACATCCTTCTCT
Pax6	GCTTCATCCGAGTCTTCTCCGTTAG
	CCATCTTTGCTTGGGAAATCCG
Sonic Hedgehog	GCCTACAAGCAGTTTATTCCCAAC
	CAGTGGATGTGAGCTTTGGATTC
T-Brachyury	TGCTGCCTGTGAGTCATAAC
	TCCAGGTGCTATATATTGCC
Wnt-4	GCGGGTGGAGTGCAAGTGTCACGGGG
	CACCGGCACGTGTGCATCTCCACGAG
WT-1	CGCACATCCTGAATGCCTC
	GAGGCCCTACAGCAGTGACAA

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