H24/3626

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 23 March 2004 .....
.....[redacted].....
Sec. Research Graduate School Committee

Supervisor's signature:
(please print name)

[redacted]

Date: 11/12/03

ELF5 is an Epithelial-Specific Member of the *Ets* Oncogene/Tumour Suppressor Gene Family

---

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Erika Jane Lapinskas

BSc. (Science Scholars Programme) (HONS)

Centre for Functional Genomics and Human Disease

Monash Institute of Reproduction and Development

Monash University

Australia

December 2003

Table of Contents

List of Figures and Tables

Abstract

ELF5 is a member of a large family of *ets* transcription factors that share a highly conserved DNA-binding domain. Most *ets* factors are expressed in the haemopoietic compartments, but *ELF5* belongs to a small epithelial-specific subfamily. *Ets* genes have historically been involved in cancers, particularly in various types of leukaemia and lymphoma. However, the most prevalent cancers affecting humans are solid tumours arising from epithelial cells. Interestingly, *Elf5* protein expression is restricted to epithelium found in the breast, kidney, salivary gland and stomach, as shown by immunohistochemistry.

Preliminary evidence implicates *ELF5* as an epithelial-specific tumour suppressor gene. *ELF5* is localised to human chromosome 11p13, a region that frequently undergoes large deletions, or loss of heterozygosity (LOH) in neoplasia, and contains tumour suppressor elements. *ELF5* may be linked to breast cancer via the *BRCA1* pathway. In MCF7 cells, *ELF5* is a novel transcriptional activator of the tumour suppressor gene *BRCA1*, which is downregulated in half of sporadic breast cancers.

Previously, *ELF5* expression was not detected in 20/24 epithelial-derived carcinoma cell lines and was presumed to be lost in these compared with normal tissue. LOH was examined as a potential cause for loss of expression. Analysis showed that the locus was not deleted in 18 breast carcinomas. *ELF5* expression is downregulated by NFκB, which is over-expressed in various carcinomas. This may provide a reason for the decrease in *ELF5* transcript in carcinoma cells.

Further *ELF5* expression analysis of 27 primary breast carcinomas by real-time RT-PCR shows that half of the cancers have increased expression, and half decreased, constrasting the *in vivo* situation with that found in cell lines. Over-production of the protein is observed in 44 tumour sections stained with ELF5 antibody. Re-expression of *ELF5* in the breast carcinoma cell line MCF7 showed no inhibition of the growth capabilities of the cell line. This situation may be paralleled in the prostate, which also over-expressed the protein in 10 carcinomas compared with normal prostate and BPH. In the kidney, however, *ELF5* mRNA was drastically reduced in 18 of 19 carcinomas

studied. Forty carcinoma sections did not stain for *ELF5* protein expression though normal kidney shows positive epithelial staining. Re-expression of *ELF5* in 786-O renal carcinoma cells demonstrated inhibition of anchorage-independent growth and tumourigenicity in immunocompromised mice, indicating that *ELF5* acts as a tumour suppressor in this organ.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference is made in the text. Where the work in this thesis is part of joint research, the relative contributions of the respective persons are acknowledged in the text.

[redacted]

Erika J. Lapinskas

Acknowledgments

I dedicate this thesis and the work therein to my Lord and Saviour, Jesus Christ. None of this could have happened without Him.

I would like to thank the Centre for Functional Genomics and Human Disease and the Director, Associate Professor Paul Hertzog, for allowing me to study in this laboratory. I appreciate greatly the assistance and advice of Dr Melanie Pritchard, throughout her tenure as my supervisor. I am also grateful to other post-doctoral researchers in the lab who have assisted me with scientific discussions and alternative perspectives on difficult experiments, particularly Dr Ernst Wolvetang, Dr Jo Tkalcevic and Dr Sika Ristevski. The ETS group has been a good forum for expression of ideas and the presentation of data, so I appreciate the input of everyone (both past and present) in this group.

I am grateful for the support system the students have provided for each other – having each other as lab-mates and friends as well has helped me greatly in getting through the tough times. Nicole Flentjar, Debbie O'Leary, Laureane Mittaz-Crettol, Jiong Zhou, Annie Ng and more recently, Renee Chehab, have all been constituents of this system. Particularly Nicole, Debbie and Laureane, I will miss you all as we depart from the lab and go our separate ways – but I don't think we'll ever forget our time here; from the meetings to the lunches to the gym workouts and 'whinge' sessions.

I wish to extend my eternal gratitude to my family and friends, whose support has helped me more than anyone can know. While I haven't always wanted to talk about it, I appreciate you asking! Thanks to my family, who have supported me in all ways and been patient with everything that this task has required of me, and for your pride in my work. Thanks to my friends both old and new, but you're all certainly life-long. Broni in particular – the mochas, the food, the holidays, the distractions! I love you all so much, and this thesis is as much for you all as it is for me.

Publications and Conference Presentations

Lapinskas, E. J., Palmer, J., Hertzog, P. J., Hammacher, A. and Pritchard, M.A. (2003) **Expression of the *ets* transcription factor Elf5 is restricted to glandular epithelium.** Under review for *Histochem & Cell Biol.*

Zhou, J., Pritchard, M.A., Wilson, T.J., **Lapinskas, E.J.,** Naylor, M. J., Harris, J., Tsao, S., Tellis, I., Zavarsek, S., Xu, D., Visvader, J., Lindeman, G. J., Thomas, R., Ormandy, C.J., Hertzog, P. J. and Kola, I. (2003) ***Elf5* is essential for early embryogenesis and mammary gland development during pregnancy and lactation.** Accepted by *EMBO J.*

Lapinskas, E. J., Palmer, J., Brown, M., Hertzog, P. J., and Pritchard, M.A. **Is *ELF5* a tumour suppressor gene?** Poster presentation, 12th Annual Lorne Cancer Conference. (Awarded Student Bursary).

Lapinskas, E. J., Palmer, J., Brown, M., Hertzog, P. J., and Pritchard, M.A. **The role of *ELF5* as an epithelial-specific tumour suppressor gene that regulates *BRCA1* in MCF7 cells.** Poster presentation, Cancer Genetics and Tumour Suppressor Genes Meeting, Cold Spring Harbor Laboratories, New York, 2002.

Lapinskas, E. J., Palmer, J., Brown, M., Hertzog, P. J., and Pritchard, M.A. **The role of *ELF5* as an epithelial-specific tumour suppressor gene that regulates *BRCA1* in MCF7 cells.** Poster presentation, 14th Annual Lorne Cancer Conference.

Lapinskas, E. J., Palmer, J., Brown, M., Hertzog, P. J., and Pritchard, M.A. **The ETS transcription factor *ELF5* is restricted to glandular epithelium and is a putative tumour suppressor in mammary epithelium.** Poster presentation, 5th International Genetics Congress, Melbourne, 2003.

Chapter 1. Literature review

1.1 Transcription factors – regulation of the transcriptome

Regulation of the transcription of the genome to determine different cell lineages; to alter cell fate in response to temporal and spatial signals during development, is largely effected by the actions of proteins known as transcription factors. A general set of transcription factors, present in all cell types in the human, is used to initiate transcription of all genes necessary for cell growth, division, differentiation and death. These proteins have the capability to promote transcription of DNA to messenger RNA and include RNA polymerase II, transcription elongation factors, other enzymes responsible for mRNA processing and the proteins that constitute the pre-initiation complex that assists in forming the three dimensional DNA structure necessary for transcription initiation (Maniatis and Reed, 2002). These enzymes bind as a holoenzyme to DNA sequence motifs found in the non-transcribed promoter regions of all genes.

Different cell types require expression of certain subsets of genes. The activity of the general transcription complex is directed by the action of other, specific transcription factors, which recognise motifs in promoters of particular downstream genes. Binding of these factors recruits the generic transcription complex and initiates transcription in a

controlled manner. The expression of specific transcription factors can be tightly regulated at the transcriptional, translational and post-translational levels and these factors function to determine the appropriate expression of genes in a given cell at any time-point.

1.2 The evolution of transcription factor families

Many classes of transcription factors have been described in *Homo sapiens*, with orthologues in other species. Generally, transcription factor families are defined by a group of proteins possessing a characteristic domain/s or set of motifs that defines them as being related evolutionarily. Different family members, by virtue of conserved protein domains, tend to have similar but not identical functions. Specificity of protein function is conveyed mainly by amino acid differences, divergent spatial and temporal expression patterns and post-translational regulation.

Multi-member transcription factor families appear to have evolved from a single ancestral gene formed at some time during the evolution of life. Some gene families have ancient ancestry, with homologues being identified in all eukaryotes. The ancestral genes of these families presumably originated before the division of the plant and animal kingdoms. Other factors, in particular components of the basal transcription machinery, display similarities even between prokaryotes and eukaryotes, indicating predictably that these genes were among the first to arise.

1.3 The Ets family of transcription factors

The *ets* transcription factor family originated relatively recently in evolutionary terms. *Ets* genes are found in all metazoans, but have not been identified in plants, prokaryotes or the budding yeast *Saccharomyces cerevisiae*. The model organism *Caenorhabditis elegans* possesses ten *ets* genes, with *lin-1*, the first of these to be identified, believed to be most similar to the ancestral *ets* gene (Hart et al., 2000b). It is most similar to the *ets-1* gene found in other species, including *Gallus gallus, Drosophila melanogaster, Mus musculus* and *H. sapiens*, the most widely studied species in *ets* biology.

1.3.1 Phylogeny of the *ets* family

The archetypal *ets* gene, *ets-1*, was first identified as the cellular counterpart of part of a chimeric viral genome from the E26 avian leukaemia retrovirus, which causes myeloblastosis and erythroblastosis in chickens (Nunn et al., 1984). The presence of the viral *ets* gene, found in the E26 genome as part of the tripartite p135 viral gene (*gag-myb-ets*), was found to correlate with the ability of the virus to cause erythroblastosis (Leprince et al., 1983). The related retrovirus 'avian myeloblastosis virus' (AMV) shares the *myb* but not the *ets* sequence, in addition to essential virion genes, but causes myeloblastosis exclusively (Nunn et al., 1983), indicating that the presence of v-*ets* is necessary for the ability of E26 to cause erythroblastosis. A cellular counterpart to the v-*ets* oncogene was cloned from transcripts found in normal chicken cells and named c-*ets*-1 (Leprince et al., 1983; Nunn et al., 1984). Subsequently, the human *ets-1* and *ets-2* loci were identified by

hybridisation on human chromosomes 11 and 21 respectively (Watson et al., 1985). Since this time, over 30 *ets* paralogues have been described in the human genome, and a lower number in less complex organisms such as *D. melanogaster* and *C. elegans* (Table 1).

The evolutionary relationship of these genes has been described by phylogenetic analysis, using the highly conserved *ets* DNA-binding domain (Section 1.3.2) as a basis for this analysis (Figure 1.1). The *ets* domain has been conserved in both sequence and function between species; the vertebrate *ets* domains of *Ets-1* and *Ets-2* are capable of rescuing the midline glia and photoreceptor development phenotype of the *pointedP2 D. melanogaster* mutant (Albagli et al., 1996). Regions outside the *ets* domain vary much more widely between family members and are likely to contribute to the differences in function discerned between the proteins. For example, most *ets* proteins transactivate target promoters, while a subset are dedicated transrepressors. This may be due to possession of autonomous transactivating or repressing domains. However, protein interactions mediated by divergent sequences outside the *ets* domain may determine whether a given *ets* protein will activate or repress in a context-dependent manner.

Table 1.1. ***Ets*** **genes identified in** ***H. sapiens, M. musculus, D. melanogaster*** **and** ***C. elegans*** **have been grouped into subfamilies based on sequence similarity within the** ***ets*** **domain.**

Ets subfamily (*H. sapiens*)	Gene name	Homologue		
		M. musculus	*D. melanogaster*	*C. elegans*
ELK	*SAP-1/ELK-4* (Dalton and Treisman, 1992) *NET/ELK-3* (Nozaki et al., 1996) *ELK-1* (Rao et al., 1989)	*Sap-1/Elk-4* (Dalton and Treisman, 1992) *Net/Erp/Elk-3* (Lopez et al., 1994) *Elk-1* (Grevin et al., 1996)		*lin-1* (Beitel et al., 1995)
ETS	*ETS-1* (Watson et al., 1985) *ETS-2* (Watson et al., 1985)	*Ets-1* (Watson et al., 1986a) *Ets-2* (Watson et al., 1988)	*pointed* (Klambt, 1993)	
ELF	*ELF-1* (Leiden et al., 1992) *NERF* (Oettgen et al., 1996) *MEF/ELF-4* (Miyazaki et al., 1996)	*Elf-1* (Davis and Roussel, 1996) *Mef/Elf-4* (Miyazaki et al.)	*e74* (Janknecht et al., 1989)	
ELG	*E4TF-1* (Watanabe et al., 1993)	*Gabpα* (LaMarco et al., 1991)	*elg* (Pribyl et al., 1991)	
ERF	*ERF* (Sgouras et al., 1995) *ETV-3/PE-1* (Klemsz et al., 1994)	*Erf* (Liu et al., 1997a) *Pe-1* (Bidder et al., 2000)		
ERG	*FLI-1* (Watson et al., 1992) *ERG* (Reddy et al., 1987) *FEV* (Peter et al., 1997)	*Fli-1* (Klemsz et al., 1993) *Erg* (Rivera et al., 1993)	*ets-3* (Chen et al., 1992) *ets-6* (Chen et al., 1992)	

PEA3	*ER81/ETV-1* (Monte et al., 1995) *ERM/ETV-5* (Monte et al., 1994) *E1A-F* (Kaya et al., 1996)	*Er81* (Jeon et al., 1995) *Pea3* (Xin et al., 1992)	*ets96B* (Hsu and Schulz, 2000)	
SPI	*PU.1/SPI-1* (Ben-David et al., 1991) *SPI-B* (Ray et al., 1992) *SPI-C* (Carlsson et al., 2002)	*Pu.1/Spi-1* (Klemsz et al., 1990) *Spi-B* (Chen et al., 1998) *Spi-C/Prf* (Bemark et al., 1999)		
YAN	*TEL/ETV6* (Poirel et al., 1997) *TEL-2* (Potter et al., 2000)	*Tel/Etv6* (Poirel et al., 1997)	*yan* (Lai and Rubin, 1992)	
ESE	*ELF3/ESE-1/ESX/ERT/JEN* (Oettgen et al., 1997a; Tymms et al., 1997) *ELF5/ESE-2* (Oettgen et al., 1999b; Zhou et al., 1998) *EHF/ESE-3* (Kas et al., 2000; Kleinbaum et al., 1999)	*Elf3* (Tymms et al., 1997) *Elf5* (Zhou et al., 1998) *Ehf* (Bochert et al., 1998)		
ETS-4	*PSE/PDEF* (Oettgen et al., 2000)	*Pse* (Yamada et al., 2000)	*ets-4* (Chen et al., 1992)	
No group assigned		*Er71* (Brown and McKnight, 1992)		

1.3.2 The Ets domain and DNA-binding specificity

Ets genes are defined by the presence of the ~80 amino acid, highly conserved, *ets* (E-twenty-six) DNA-binding domain. This domain mediates binding to the *ets* binding site (EBS), a DNA target with a purine-rich GGAA/T core (Karim et al., 1990; Wasylyk et al., 1992) found in the promoter and enhancer elements of many genes. All known *ets* proteins bind these motifs, which, though similar, are not identical. *Ets* proteins are crucial for regulating target genes involved in cell growth, differentiation and apoptosis. Addition of synthetic *cis* elements upstream of a minimal promoter, and the subsequent transfection of these constructs into the neuroblastoma cell line Neuro2A, showed that in constructs with increased promoter activity, eight motifs predominated - AP2, CEBP, GRE, Ebox, ETS, CREB, AP1, and SP1/MAZ. Notably, the most active constructs constituted combinations of these binding sites (Edelman et al., 2000), indicating that *ets* binding sites are some of the most important for target gene transcriptional regulation.

Despite the large number of *ets* genes and downstream target promoters, functional redundancy is not widespread within the *ets* family, as demonstrated by the different phenotypes of targeted null mice, *D. melanogaster* and *C. elegans* (Section 1.4). For example, crosses of heterozygote *PU.1* mice with null *Spi-B*, *Ets-1* or *Elf-1* mice demonstrate that there is no genetic overlap between *PU.1* and *Ets-1* or *Elf-1*, but that there is a partial overlap between *PU.1* and *Spi-B* target genes, as the $PU.1^{+/-}/Spi\text{-}B^{-/-}$ mice show additional B cell defects. Therefore, *PU.1* and *Spi-B* coordinately regulate a subset of targets but do not overlap with other haematopoietically-expressed *ets* genes (Garret-Sinha

et al., 2001). This biological specificity is due to several mechanisms that select distinct target genes for each *ets* factor.

The specificity of protein-DNA interaction may be mediated by a variety of mechanisms. Firstly, differences in sequence of the *ets* DNA-binding domain result in different preferential contacts in the major groove of the central *ets* motif, with minor groove interactions predicted in the flanking nucleotides (Nye et al., 1992). In T cells, the closely related *Ets-1* and *Elf-1* are highly expressed but regulate different sets of target genes. *In vitro,* Ets-1 preferentially binds the TCRα and TCRβ *ets* sites but not either of two sites in the IL-2 enhancer. Elf-1, however, displays a distinct binding preference for sites in the IL-2 and HIV-2 enhancer elements. A comparison of *ets* binding sites identified three flanking nucleotides 3′ to the GGA trinucleotide core that accounted for the differential binding of the two proteins (Wang et al., 1992). Likewise, the haemopoetic proteins *Ets-1* and *PU.1* have different binding specificities and transactivate through distinct motifs. The binding specificity appears to be controlled by a combinatorial effect of the nucleotides flanking the *ets* motif rather than any single base pair (Wasylyk et al., 1992). However, the PU.1-related protein Prf/Spi-C, expressed predominantly in B cells, interacts with PU.1 binding sites in a variety of B-lineage promoters including the Igκ 3′ enhancer and may antagonise PU.1-dependent transactivation. Its ability to bind these sites is presumably due to its high level of amino acid identity (57%) with the murine PU.1 *ets* domain (Bemark et al., 1999; Hashimoto et al., 1999).

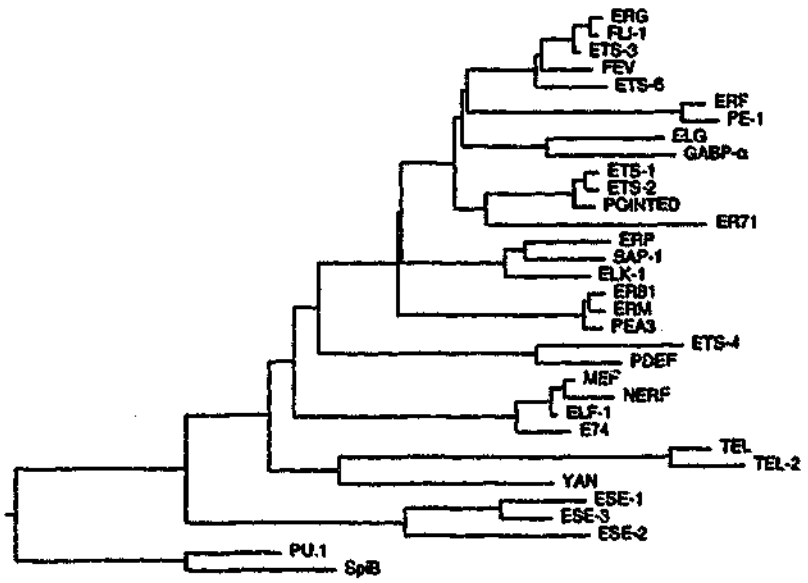


Figure 1.1. Phylogenetic analysis of the *ets* family based on amino acid sequence similarity within the *ets* domain. Branch length of the inferred phylogenetic tree is based on degree of similarity between members, where short braches indicate highly similar homologues. Human and *Drosophila* homologues only are included for simplicity. For Genbank accession numbers, see Table 1. (Adapted from Kas et al., 2000).

Variations in the flanking nucleotides surrounding the *ets* motif in gene promoters are therefore selective for binding by different *ets* proteins. In sea urchin embryos, targeted alterations in the sequence of an EBS resulted in a change in preference for the *ets* factor binding to that site. Three base pairs near the GGA *ets* motif in the *CyIIa* gene promoter were switched, thus converting it to another type of *ets* site found in the hatching enzyme promoter. Expression of the altered *CyIIa ets* site fused with a heterologous basal promoter was sufficient to change the expression pattern of a reporter gene from mesenchyme to ecto- and endodermal expression, mimicking the expression of the hatching enzyme (Consales and Arnone, 2002). This is consistent with the hypothesis that binding affinity of *ets* proteins to particular DNA motifs is important in determining temporal and spatial gene regulation. Similarly, in the mouse α4 integrin promoter, the arrangement of three *ets* binding sites is critical for transcription of the gene. Of the three sites, the 3′ site bound the GABPα/β complex whereas the other two were required for formation of another cell-type specific *ets*-containing complex which correlates with a high level of α4 integrin transcription. Deletion of the 5′ site resulted in the loss of this *ets*-containing complex, but the formation of alternative *ets*-containing complex that substituted for its action (Rosen et al., 1994). This demonstrates that co-ordinate action of *ets* proteins on DNA is also important for transcriptional regulation of target genes, rather than simply selection of a single *ets* protein by a particular *ets* motif.

Secondly, the cell-type specific expression patterns of different *ets* proteins must also determine which potential target genes are relevant *in vivo*. Blood-specific *ets* proteins

such as PU.1 and Elf-1, for example, may be capable of binding *ets* motifs in the promoters of epithelial-specific downstream targets, but this interaction would be irrelevant *in vivo*, where the *ets* protein is not present in the cell.

Thirdly, regulation of target genes may be mediated by interaction of *ets* factors with other transcription factors on DNA. One pertinent example is co-operation of *ets* proteins with the c-fos/c-jun dimer AP-1, as *ets* and AP-1 motifs are frequently found in close proximity in promoter regions. The PE-1 protein, expressed in osteoblasts among other cell types, suppresses transcription of matrix metalloproteinase-1 (MMP-1) in response to fibroblast growth factor receptor (FGFR) signalling. An *ets* binding site was identified in the proximity of the AP-1 site in this promoter (Lamph et al., 1988). PE-1 appears to interact with AP-1 to prevent DNA-protein interaction on the AP-1 site in the MMP-1 promoter (Bidder et al., 2000). However, other *ets* factors also interact with family members of the AP-1 subunit c-Jun to potentiate or abrogate AP-1 mediated transactivation of MMP-1. In NIH3T3 fibroblasts, Ets-1 augmented MMP-1 promoter activity 10-fold with c-Jun or Jun-B, whereas Fli-1 enhanced only Jun-B-mediated induction. PU.1, however, prevented promoter induction by both c-Jun and Jun-B (Westermarck et al., 1997), indicating again the specific roles the *ets* transcription factors play in co-ordinate promoter regulation. Another important *ets* protein:protein interaction is that of Ets-1 and Pax-5 interaction on the *mb-1* promoter, where Pax-5 recruits Ets-1 upon DNA binding. The interaction with Pax-5 increases Ets-1 DNA binding specificity to the suboptimal motif GGAG (Garvie et al., 2001). This discovery opens up a new set of potential target genes for regulation by *ets* proteins, and also a new mechanism for generating specificity of *ets* binding to DNA.

Finally, *ets*-binding proteins may compete against unrelated transcription factors for binding to their cognate motif in a concentration-dependent manner. Basal transcription of the human folate receptor (hFR) type γ gene is driven by a canonical Sp1 element, but transcription is enhanced by an upstream *ets* binding site that overlaps a second, non-canonical, Sp1 element. This element is bound with similar affinity to the first. Using cell lines expressing varying levels of Sp1, luciferase reporter assays showed that this promoter was competitively repressed by high concentrations of Sp1 due to binding of Sp1 to the dual EBS/Sp1 site. Where the Sp1 concentration was low, Ets-1 protein bound the EBS and synergised with Sp1 on the downstream element to enhance transcription of the hFR-γ promoter (Kelley et al., 2003). The authors also demonstrated that Sp1 could bind a variety of EBS, including those responsive to Ets-1 and Spi1/PU.1. This indicates a novel, concentration-dependent mechanism for the activation and repression of Sp1/Ets regulated promoters that may contribute to tissue-specific and temporal gene regulation. It is not known, but predictable, that similar mechanisms could exist for other transcription factor pairings.

There is experimental evidence that all of the above mechanisms form the basis for *ets* target gene specificity *in vivo*. It is likely that they work in a combinatorial fashion but that they are active to different degrees depending upon the context that includes the cell type, the *ets* motif sequence, and of course the relevant *ets* protein and the protein partners with which it interacts.

1.3.3 The Pointed (pnt) domain

The *ets* family has been further divided into sub-families, which are also defined by similarity within the *ets* domain (Figure 1.1). Clustering the genes on this basis, as expected, correlates with conservation of additional sequence motifs within subfamilies. The most prominent example of this is the pointed domain (*pnt*) found in the *Ets, Elg, Erg, Tel/yan,* and *Ese* subfamilies as well as *Pse/Pdef* (Chang, 1997; Graves and Petersen, 1998; Kas et al., 2000; Neve et al., 1998; Oettgen et al., 1999a; Tugores et al., 2001; Tymms et al., 1997; Yamada et al., 2000; Zhou et al., 1998) (Figure 1.2). This region, approximately 80 amino acids in length, has also been called the 'B-box' (Hill et al., 1993; Treisman et al., 1992) or sterile alpha motif (SAM) domain (Jousset et al., 1997; Stapleton et al., 1999). It has been shown to function in protein-protein interactions. The *pointed* domain was described by sequence similarity to the *D. melanogaster ets* gene *pointed,* which encodes two *ets* proteins required for glial-neuronal cell interactions at the ventral midline (Klambt, 1993).

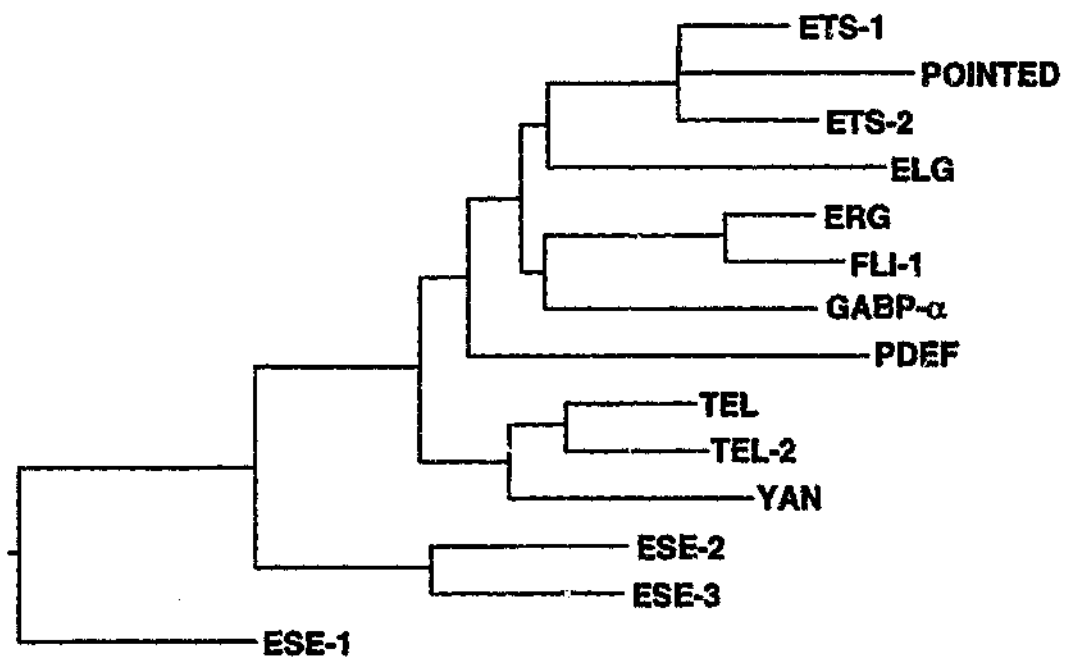


Figure 1.2 Phylogenetic analysis of *ets* domain factors based upon amino acid sequence identity within the *pointed* domain. Thus only subfamilies possessing the *pnt* domain are shown. The phenogram representation of the inferred phylogenetic tree is shown. Branch lengths indicate relative similarities between *pnt* domains, where short branch lengths indicate highly similar homologues. For simplicity, only human and *Drosophila ets* factors are included. For Genbank accession numbers, see Table 1. (Adapted from Kas et al., 2000)

In Ets-1, the *pnt* domain forms four core α-helices with an additional N-terminal helix (Slupsky et al., 1998) suggesting that it functions in interacting with other proteins. The *Erg pnt* region dimerises with itself and with two shorter Erg isoforms, and can also heterodimerise with other *ets* proteins, Fli-1, Ets-2, Er81 and PU.1 (Carrere et al., 1998).

The *pnt* domain is not the only domain through which *ets* proteins can interact with others. Individual factors have a collection of unique interaction domains. For example, an amino-terminal domain found in the Tel protein is unique in that it serves as an oligomerisation interface for Tel. Swapping this domain with the homologous region of Ets-1, Ets-2 or GABPα results in loss of this capability, which accounts for the oncogenic interaction of Tel-platelet-derived growth factor β (PDGFβ) fusion proteins in t(5;12) chronic myelomonocytic leukaemia cells and thus constitutive protein kinase activity (Jousset et al., 1997). While these unique *ets* protein-specific domains exist in various family members, common themes are found in the conserved regions of the proteins – indeed, even the ets domain itself can mediate protein:protein interaction (Section 1.3.6).

1.3.4 Cis-acting autoinhibitory domains

Several *ets* transcription factors possess cis-acting autoinhibitory domains that prevent DNA-binding. These include Ets-1 (Donaldson et al., 1996; Skalicky et al., 1996; Wasylyk et al., 1992), Elk-1 (Janknecht et al., 1994; Yang et al., 2002), Net (Criqui-Filipe et al., 1999; Maira et al., 1996), PU.1 (Brass et al., 1996), ER71 (de Haro and Janknecht, 2002), PEA3 (Greenall et al., 2001) and other members of the PEA3 subfamily (Brown et al., 1998; Laget et al., 1996; Xin et al., 1992) and possibly ELF5 (Oettgen et al., 1999b).

Provision of autoinhibitory modules that block DNA-binding and therefore transcriptional activation may be crucial for *ets* protein regulation, given that the high degree of conservation within the family could result in binding to inappropriate *ets* sites. Therefore, triggers must also exist that reverse the autoinhibition and allow recruitment of proteins to the appropriate site at the correct time. These triggers, and the type of autoinhibitory domain, vary between factors depending on the cellular pathways they act in.

In the PEA3 protein, DNA-binding activity is inhibited by a combination of two motifs located on either side of the *ets* domain. Relief of the inhibition requires interaction with the USF-1 protein, which may modify the protein structure to promote association of the DNA:protein complex (Greenall et al., 2001). In the ternary complex factor Elk-1, which possesses a novel repression domain related to the p300 CRD inhibitory domain, autoinhibition is relieved upon phosphorylation by mitogen-activated protein kinases (MAPK) (Gille et al., 1995; Yang et al., 2002; Yang and Gabuzda, 1999).

Similarly, Ets-1 DNA-binding activity is inhibited by the presence of α-helices lying N- and C-terminal to the *ets* domain. Interaction with proteins such as AML-1/CBFα2 appears to disrupt autoinhibitory structures on both proteins, allowing high-affinity DNA binding (Goetz et al., 2000; Gu et al., 2000; Jonsen et al., 1996; Petersen et al., 1995). Deletion of this module, as found in a natural alternative splice product of human ETS-1, causes higher affinity binding to a target sequence, probably because the DNA-binding domain structure is not obscured by the presence of the autoinhibitory module (Wasylyk et al., 1992).

Further mechanisms of inhibition and activation of *ets*-dependent transactivation are still being discovered. *Ets-1* binds a palindromic head-to-head *ets* motif in the stromelysin (MMP3) promoter co-operatively, where one Ets-1 molecule binds to one EBS, and then facilitates binding of a second Ets-1 molecule to the other EBS. Co-operative binding is dependent on the N-terminal of the protein, as shown by construction of N-terminal deletion mutants, and requires the counteraction of autoinhibition that is dependent on the residues 245-330 encoded by exon VII of the gene (Baillat et al., 2002). The natural shorter p42 isoform of Ets-1, which deletes exon VII, did not bind the palindrome cooperatively and hence showed decreased transactivation on the stromelysin promoter. Therefore, there is more than one route for control of autoinhibition within the *ets* family. Other members of the family, particularly the recently discovered epithelial-specific *ets* (ESE) subfamily, possess putative autoinhibitory domains but the manner in which they function has not yet been elucidated.

1.3.5 A subset of ets factors are regulated by the MAP kinase pathway

As with all transcription factors, *ets* proteins are the downstream effectors of signalling cascades. A subset of *ets* genes contains MAPK sites and is regulated by this pathway. The effects of intracellular signalling pathways affect particular *ets* factors at more than one level: by altering their subcellular localisation (Ducret et al., 1999) and/or their ability to bind DNA and/or to transactivate promoters by interaction with other transcription factors (Criqui-Filipe et al., 1999; Ducret et al., 1999; Jacobs et al., 1998).

Firstly, the *D. melanogaster ets* transcription factor *pntP2* is phosphorylated by MAPK at a single site, resulting in transactivation of downstream target genes in the R7 photoreceptor neuron differentiation pathway (Brunner et al., 1994). A *D. melanogaster ets* protein with transcriptional repressor activity, yan, is activated by the same signalling pathway but negatively regulates the same targets (Lai and Rubin, 1992). Thus, signals communicated by the MAPK pathway can be integrated by multiple *ets* factors at the levels of target gene transcription. This is also apparent in the human and murine Elk ternary complex factor (TCF) subfamily, where phosphorylation by ERK, one of the five major MAPK pathways, initiates ternary complex formation by Elk-1 (Gille et al., 1992; Janknecht et al., 1994).

In the complex mammalian context, regulation of *ets* by MAPK cascades may be more involved, leading to different molecular end-points via the same factor, as *ets* proteins may be targets of more than one MAPK cascade. An example is the Elk-1-related TCF, Net, where phosphorylation by the ERK pathway triggers loss of autoinhibition and therefore activates the protein (Criqui-Filipe et al., 1999), while phosphorylation by the JNK pathway results in export of Net from the nucleus and therefore loss of activity (Ducret et al., 1999). The mammalian homologues Ets-1 and Ets-2 are also regulated post-transcriptionally by phosphorylation in response to mitogenic signalling (Fujiwara et al., 1990; Pognonec et al., 1990). When a conserved ERK2 docking site in the Ets-2 *pnt* domain is phosphorylated, it enhances the efficiency of ERK interaction with a second phosphorylation site N-terminal to the *pnt* domain, enhancing Ras-mediated potentiation of ets-2 transactivation (Seidel and Graves, 2002). However, MAP kinase signalling does not affect Ets-1 DNA-binding activity. Rather, calcium-dependent phosphorylation of Ets-1 on serine residues close by

the autoinhibitory domain, can reinforce inhibition of DNA-binding activity (Cowley and Graves, 2000). This demonstrates that while *ets* factors are regarded as being regulated most frequently by MAPK cascades, other signals are also crucial to regulate their activity.

While most *ets* proteins activate transcription, three thus far have been shown to act as transcriptional repressors in a mammalian system: the yan homologue Tel, Erf and Net. These possess distinct transrepression domains and, at least for Net and Tel, transrepression may be effected by recruiting histone deacetylase (HDAC). Erf physically associates with, and is phosphorylated by ERK upon Ras/ERK signalling, resulting in exportation of Erf from the nucleus (Le Gallic et al., 1999). Phosphorylation by ERK therefore decreases Erf repressor activity (Sgouras et al., 1995). Mutation of the ERK phosphorylation sites to alanine results in Erf protein that is not ERK phosphorylated, is constitutively nuclear and, unlike wildtype Erf, can block Ras-mediated transformation of NIH3T3 cells. This also indicates that Erf is a physiological target of the Ras-ERK pathway (Mavrothalassitis and Ghysdael, 2000).

The recently discovered epithelial-specific *ets* (ESE) subfamily may also be MAPK substrates. The ESE-3 protein, for example, is a nuclear effector of MAPK signalling, and upon Ras signalling co-operates with AP-1 to repress target genes in a context-dependent manner (Tugores et al., 2001). ELF5 contains consensus protein kinase C and tyrosine kinase recognition sites but it is not known which pathway activates these (Zhou et al., 1998). The molecular end-points that may result from phosphorylation of these factors by MAPK cascades have not been studied extensively.

1.3.6 Protein interactions and Ets family members

Identification of protein interacting partners for *ets* proteins has been the focus of a large body of recent research. For transcription of downstream target genes to occur, it is essential for DNA:protein interactions to be established. However, protein:protein interactions are also necessary, from the association of specific transcription factors with the basal transcription machinery to interaction with other non-DNA binding transcription-related proteins. *Ets* factors that possess a *pnt* domain are capable of heterodimerising with other *ets* proteins, and in some cases homodimerising. However, each *ets* protein has further unique protein interaction domains specific to its own function.

Much of the early work regarding *ets* protein interactions involved the haematopoietic-expressed Tel protein, given its role as an oncogenic effector after chromosomal translocation in some leukaemias (Golub et al., 1995). Via its *pnt* domain, Tel can bind itself and also to another *ets* family member, Fli-1, both *in vitro* and *in vivo*. By binding Fli-1, Tel inhibits its transcriptional activity and therefore its action on downstream megakaryocyte-specific promoters (Kwiatkowski et al., 1998), indicating the importance of *ets-ets* interactions in regulation of target promoters. Further experiments focused on identifying *ets* interactions with unrelated proteins and demonstrated a collection of potential interacting partners for the closely related *Xenopus laevis* (*X. laevis*) Fli-1 and Erg proteins. These were identified using the yeast-two-hybrid (Y-2-H) system. Fli-1 and Erg partnered with two homeobox transcription factors, RNA splicing and RNA binding proteins and a protein associated with haematopoiesis, and Erg with the ubiquitous heat shock protein 90 (Deramaudt et al., 2001). Erg also forms homodimers and heterodimers

with other *ets* transcription factors and with the AP-1 complex (Carrere et al., 1998). This implicates Fli-1 and Erg proteins in development, haematopoiesis and cellular stress responses both through interaction with other transcription factors on promoters and through a possible new role in mRNA processing. Further Y-2-H studies have shown that Fli-1 also interacts with the erythroid and megakaryocytic differentiation-related zinc finger transcription factor, GATA-1. The *ets* domain of Fli-1 physically interacts with the zinc finger domain of GATA-1. In the presence of DNA containing *ets* and GATA-1 binding sites, this translates to co-operative binding and transcriptional synergy (Eisbacher et al., 2003).

In contrast, the activity of the GABP complex is dependent upon the interaction of two invariant subunits, GABPα and GABPβ. The binding of the *ets* domain protein GABPα with its partner, the non-DNA binding GABPβ, occurs via an ankyrin repeat sequence and is crucial for the transcriptional activity of the complex. These proteins assemble into a tetramer that can subsequently bind DNA with high affinity (Brown and McKnight, 1992). This does not preclude interaction with other proteins; on the *Pem* homeobox gene promoter, GABP transactivation is dependent upon an intact Sp1 binding site, indicating the necessity to co-operate with an Sp1 family member in this context (Rao et al., 2002).

Therefore, as discussed above, a major factor in both the specifity and affinity of *ets* factor binding to DNA is mediated by interaction with its protein partners. *Ets* proteins do not bind with high affinity to DNA alone, but act as members of multiprotein complexes. This is exemplified by the protein partnership of Ets-1 and Pax-5 on DNA. Unlike previous

studies examining protein:protein interactions in the context of the GGAA/T DNA motif, Garvie et al. (2001) showed that when Ets-1 interacts with Pax-5 in context with DNA, Ets-1 structure is altered, enabling it to interact with non-*ets* consensus DNA motifs. These data indicate that the range of potential downstream *ets* target genes may be much greater than expected as the GGAA/T *ets* consensus motif is not required for all *ets* activity. Likewise, the ternary complex factors Elk-1, Sap-1 and Net interact on the c-fos serum response element but can also bind autonomously to *ets* motifs, indicating that there is at least two sets of target genes for this subfamily (Janknecht et al., 1993). PU.1 has also been implicated in ternary complex formation with the interferon responsive factor, IRF-4. Co-operation between these two transcription factors involves DNA-binding domain association and interaction with DNA elements such as those found in the κ light chain enhancer, $E\kappa_3$ and λ light chain enhancers, Eλ2-4 and Eλ3-1 (Escalante et al., 2002).

Recent molecular modeling and structural studies (Fujii et al., 1999; Garvie et al., 2001; Hassler and Richmond, 2001; Mo et al., 2001; Verger et al., 2001; Wheat et al., 1999) demonstrate that while *ets* proteins partner with many different proteins as described above, these interactions lead to a common architectural scaffold critical for governing the specificity of DNA binding, and results in the synergistic transactivation or repression of target genes.

1.3.7 Regulation by alternative splice products

A common mechanism employed by *ets* transcription factors, among other gene families, is diversifying the function of any one gene by transcribing different isoforms from it. A

variety of examples have been studied in the *ets* family, where most members have at least two splice isoforms identified. These include the description of distinct functions for the three alternative splice isoforms generated from the *Elf-1* related factor *NERF (ELF2),* known as nerf-1a, nerf-1b and nerf-2. They differ in their amino-termini and are probably transcribed from two different transcription initiation sites. The three transcripts are differentially expressed in haematopoietic tissues and have demonstrably different functions; nerf-2 transactivates the haematopoietic *lyn* and *blk* promoters whereas the 1a and 1b isoforms do not (Oettgen et al., 1996).

Human *Erg* has at least five isoforms; Erg-1, Erg-2, $p55^{Erg}$, $p49^{Erg}$ and $p38^{Erg}$. These result from differential mRNA splicing and also usage of alternative translational initiation codons. Two of these isoforms, $p55^{Erg}$ and $p38^{Erg}$ are selectively expressed in endothelial cells, indicating a function in regulating endothelial-specific genes that is not relevant to the other three isoforms (Hewett et al., 2001). The closely related factor, Fli-1, also possesses a second isoform, Fli-1b, that is differentially expressed in haematopoietic cell lineages (Klemsz et al., 1993; Zhang et al., 1995a).

One potential reason for the differing functions of isoforms is that they are no longer affected in the same way by intracellular signalling cascades. In contrast to the major Ets-1 protein, p63, a naturally occurring p52 isoform that retains DNA-binding activity and chromatin association is not affected by calcium ionophore-dependent phosphorylation (Pognonec et al., 1990). Splicing out of recognition sites in the proteins, or altered conformation explains this important regulatory phenomenon.

Among the epithelial-specific *ets*, alternative splice isoforms have also been identified. Murine *Elf5* has two full-length transcripts that utilise alternative 3′ untranslated regions (UTRs). One of these contains motifs consistent with rapid mRNA turnover, while the other is predicted to be more stable (Zhou et al., 1998). Surprisingly, despite 95% amino acid identity between human and murine loci, human *ELF5* transcribes one isoform from the exon 1 conserved between mouse and human, and translation is initiated from exon 2. However, a second isoform is transcribed from an alternative exon 1 with an internal translation initiation codon. The transcript therefore has a different 5′UTR and 10 additional N-terminal amino acids (Oettgen et al., 1999b). A third isoform splices out exons 4 and 5 of the transcript, resulting in loss of the *pnt* domain (Appendix VII). The functional relevance of these alternative transcripts is not yet known. There is no evidence for alternative 3′ UTR usage in the human gene, but the human 3′ UTR also contains ATTAA motifs associated with rapid mRNA turnover (Oettgen et al., 1999b).

1.4 Targeted disruption of ets factors in mice

Targeted gene disruption 'knock-outs' and over-expression 'transgenic' models of *ets* factors have contributed to understanding the biological functions of these genes. Male mice lacking PEA3 exhibit sexual dysfunction, thought to arise from a neurological deficit rather than gonadal problems (Laing et al., 2000). Truncation mutants of the closely related ER81 fail to develop functioning connections between group 1a sensory afferent and motor neurons (Arber et al., 2000). Mice over-expressing dominant-negative Erm showed a

decreased ability in neuronal fate decision in neural crest cells. Glial differentiation was normal, but proliferation was impaired without affecting glial survival (Paratore et al., 2002).

Deletions of haematopoietically expressed PU.1 result in embryonic lethality due to failure of various blood-cell lineage committment (McKercher et al., 1996; Scott et al., 1994) while targeting the B cell gene *Spi-B*, as expected, causes an apoptotic response of B cells upon B cell receptor (BCR) stimulation (Su et al., 1997). *Net* knockouts die as neonates due to chylothorax, or the accumulation of lymph-derived chyle surrounding the lungs, resulting in respiratory failure. This is consistent with a transcriptionally repressive role for Net in development of the lymphatic system (Ayadi et al., 2001). Tel is required for mouse development, as null mutants die at embryonic day 10.5-11.5 (E10.5-E11.5) due to failure in yolk sac angiogenesis. No switch to bone marrow based haematopoiesis occurs in embryos, indicating a homing defect in blood cell progenitors (Wang et al., 1998). In addition, inappropriate apoptosis occurs in specific regions of the embryo, indicating that Tel is involved in non-haematopoietic lineage development in the embryo (Wang et al., 1997). *Ets-1* deficient mice develop B and T cells, but have drastically decreased numbers of natural killer (NK) and NK-T cells, and those present seem to be functionally compromised (Barton et al., 1998; Walunas et al., 2000). *Fli-1* knockouts also exhibit lethality at E12.5 due to haemorrhage and an arrest in maturation of haematopoietic progenitors (Hart et al., 2000a). Ets-2 is required for implantation, as null embryos are not detectable at day 8.5 of pregnancy. Failure of ectoplacental cone development and

trophoblast migration and defective MMP-9 expression were observed (Yamamoto et al., 1998).

Transgenic mice that over-express Ets-2 partially phenocopy Down's syndrome, which is consistent with the location of human *ETS-2* on chromosome 21 (Sumarsono et al., 1996). Additionally, *Fli-1* transgenics exhibit autoimmunological renal disease, and ultimately renal failure, associated with accumulation of certain B and T cell subsets (Zhang et al., 1995a).

Null mutants of epithelial-specific *ets* factors have also provided data indicating an important developmental role for these factors. Surviving knockouts of *Elf3* demonstrate disorganisation of the epithelium lining the small intestine. This is also associated with loss of expression of the tumour suppressor and *Elf3* target gene, *transforming growth factor β type II receptor* (*TGFβRII*), specifically in the enterocytes (Ng et al., 2002). *Elf5* null mutants die *in utero* between E3.5-7.5, possibly due to failure of trophoblast development in the null blastocysts, preventing implantation (Appendix VII).

Analysis of mouse models with abrogated expression, or over-expression, of *ets* factors has shown that these proteins have diverse and critical functions regulating genes essential for normal development. In particular, loss of an *ets* factor usually results in severe defects in the tissue type in which it should be expressed. The phenotype that ensues is frequently not compatible with fertility and/or viability.

1.5 Ets factors are involved in cancer

A collection of cellular genes known as proto-oncogenes and tumour suppressor genes are involved in controlling cell differentiation and growth processes, including growth stimulation or inhibition, mediation of signal transduction and regulation of genes responsive to these pathways. Therefore, these genes usually encode hormones and their receptors, protein kinases or DNA-binding transcription factors. Inappropriate expression of these genes, or mutations in the protein products, is thought to contribute to the development of malignancy. Transcription factor families including *jun*, *fos*, *myb*, *myc* and *ets* have been implicated in sequence-specific regulation of downstream target genes that influence the development of human malignancy (Lewin et al., 1991).

1.5.1 Oncogenic ets factors

The first association of *ets* with cancer arose from the discovery of v-*ets* in the E26 retrovirus, which transforms erythrocytes and myelomonocytic precursor cells in chicken. Following dissection of the fused nuclear oncoprotein gag-myb-ets encoded by the virus (Section 1.3.1), it was shown that the *ets* protein was necessary for erythrocytic transformation, whereas v-myb was not required, and that the DNA-binding domain of v-ets was required for myelomonocytic transformation (Domenget et al., 1992). Therefore, the cellular progenitor of v-*ets*, c-*ets*-1, was further investigated for involvement with cancer. Leukaemias were specifically investigated, given that *ets-1* is expressed in haematopoietic tissues, particularly in developing and mature T cells (Bhat et al., 1989).

Association of *Ets-1* with leukaemiogenesis, in addition to *in vitro* experiments demonstrating that over-expression of *Ets-1* in NIH3T3 cells induces colony formation in soft agar and tumour growth in nude mice, confirmed the oncogenic potential of this gene (Seth and Papas, 1990). More recently, aberrant expression of *ETS-1* in ovarian epithelial carcinoma was found to correlate with higher clinical stage, histological grade and poor prognosis (Takai et al., 2002). Similar conclusions have been drawn from studies showing over-expression of *ETS-1* in gastric carcinoma (Nakayama et al., 1996; Tsutsumi et al., 2000). Ets-1 is also required for the EGF-induced expression of the limiting telomerase component, hTERT, indicating that reconstitution of telomerase activity in cancer cells may be *ets*-related also (Maida et al., 2002). Studying *Ets-1* in the context of knockout mouse models also demonstrated the involvement of *Ets-1* in angiogenesis during development. *Ets-1* mRNA and protein levels were subsequently found to be induced by estrogen (17β-estradiol; E_2) in an *in vitro* angiogenesis model where mouse mammary tumour cells were co-cultured with rat aortic endothelial cells. This resulted in changes in *Ets-1* target gene transcription, including MMPs, vascular endothelial growth factor (VEGF) receptor, and Flt-1, favouring an invasive phenotype for the endothelial cells (Lincoln et al., 2003).

Similar *in vitro* and *in vivo* experimental models have been undertaken for a variety of other *ets* family members including *Erg* (Hart et al., 1995), *Ets-2* (Huang et al., 1994) and *Tel* (Ritchie et al., 1999), which are potentially oncogenic. Other *ets* genes have been assigned as oncogenes based upon their aberrant expression in human cancers, or participation in a cancer-associated fusion protein. The mechanism for *ets*-related tumourigenesis is not well understood, but is likely to be due to inappropriate regulation of

downstream target genes that affect parameters such as cell growth, cell cycle progression, differentiation and adherence to extracellular matrix (ECM) proteins. Examples supporting this hypothesis include that increased E1AF/PEA3 expression in mouse fibrosarcoma cells induces the metastasis-related gene *membrane-type 1 MMP* (*MT-1-MMP*) and therefore contributes to an invasive cellular phenotype (Habelhah et al., 1999). *PEA3* has been implicated as a second *ets* transcription factor whose expression in the stroma is an independent predictor of poor survival outcome in ovarian cancer. Its expression in these cases is paralleled with the cancer-associated genes β_1 *integrin* subunit, *basic fibroblast growth factor* and the MMP-inducer, *EMMPRIN* (Davidson et al., 2003). Studies of the *PEA3* subfamily in mouse mammary gland and mammary epithelial cell lines demonstrate involvement of these genes with the Wnt signalling pathway. These transcription factors interact with Wnt on the *Twist* promoter, synergistically activating *Twist* and abolishing the ability of mammary epithelial cells to induce milk protein genes in response to lactogenic signalling. This indicates that *PEA3* expression inhibits the terminal differentiation of mammary epithelia via the Wnt pathway, which is a hallmark of oncogenesis (Howe et al., 2003). *PEA3* over-expression has been reported in primary and advanced mammary lesions (Trimble et al., 1993) and expression of a dominant-negative isoform in mice inhibited Her2/*neu*-induced tumour formation (Shepherd et al., 2001), indicating an oncogenic role. Presence of PEA3 protein was a predictor of poor survival outcome in both clinical breast cancer specimens (Kinoshita et al., 2002) and in ovarian cancer (Davidson et al., 2003). In another example, in t(8;21) acute myeloid leukaemia, the AML-1/ETS fusion protein appears to interfere with MEF regulation of myeloid differentiation genes,

potentially contributing to the dedifferentiation and proliferation of the cancerous cells (Mao et al., 1999).

Aberrant regulation of *ets* genes is also biologically relevant, given that several have also been associated with specific translocations or other errors in primary cancers. For example, translocation of *ERG* t(21;22) to form a fusion protein with *EWS* in a proportion of Ewing's sarcoma cases has been reported (Sorensen et al., 1994). Four other *ets* genes have also been found fused to EWS in Ewing's sarcoma – most commonly *FLI-1* (Delattre et al., 1992), and less frequently *E1AF/PEA3* (Kaneko et al., 1996; Urano et al., 1996), *ETV-1* (Jeon et al., 1995) and *FEV* (Peter et al., 1997). All of these fusions have been found to promote oncogenesis *in vitro*. These fusion oncoproteins may contribute to oncogenesis by inappropriately regulating downstream targets, including induction of the invasion-related gene, tenascin-C (Watanabe et al., 2003). The crucial tumour suppressor, TGFβRII, is downregulated by the EWS-ets fusions, leading to TGFβ insensitivity in cancer cells (Im et al., 2000). Additionally, a t(5;12) *TEL:PDGFRβ* has been associated with progression of a subgroup of chronic myelomonocytic leukaemias to acute myelogenous leukaemia (Golub et al., 1994). Transgenic mice expressing this fusion exhibit a myeloproliferative disorder that in 20% of cases progresses to myeloid or lymphoid leukaemia (Ritchie et al., 1999).

Inappropriate expression of some of these *ets* genes, rather than translocations, has also been noted in certain cancers. Expression of the haematopoietic *ets* proteins ELF-1 and FLI-1 has been detected in the nuclei of a majority of prostate adenocarcinomas and may be

associated with activation of the metastasis-related genes urokinase plasminogen activator and its receptor (Gavrilov et al., 2001).

Activation of *ets* genes by retroviral integration is also a possible explanation for aberrant expression of *ets* in cancer, but is only apparent in two cases. In mice, *Fli-1* is activated in erythroleukaemias induced by the Friend murine leukaemia virus, due to proviral insertion in its genomic locus. Its expression inhibits apoptosis and differentiation in the affected erythroblasts, and promotes their proliferation (Pereira et al., 1999). *PU.1* is the target of insertional activation in tumours caused by the murine spleen focus forming virus (SFFV) (Moreau-Gachelin et al., 1989; Moreau-Gachelin et al., 1988).

1.5.2 Tumour suppressors and ets proteins

Tumour suppressor genes are defined by possession of one or more of a certain set of characteristics. Firstly, tumour suppressor genes are non-functional in cancer compared to normal tissue. Mechanisms of inactivation may include mutation, LOH, promoter mutation resulting in loss of transcript production or inappropriate methylation of the promoter, also associated with gene silencing. Secondly, in *in vitro* and *in vivo* assays, the gene product may be able to reverse some of the phenotypes of cancer cells. For example, re-expression of tumour suppressors in cancerous cells may repress growth, metastatic or invasive capability or growth factor independence in soft agar or nude mouse assays (reviewed in Balmain, 2002). For example, the p53 and Rb tumour suppressors regulate the cell cycle and DNA-damage responses. Changes in their coding regions, or loss of expression, can contribute to cellular transformation. Consequently, reconstituting expression of a wild-

type copy of the gene would be expected to revert the tumourigenic potential of the cells. Thirdly, genes mutated in kindreds with a hereditary pre-disposition to cancer may be tumour suppressors. For example, about half of breast-and-ovarian cancer kindreds contain a mutation in the BRCA1 tumour suppressor. The risk for developing breast cancer by age 80 for carriers (73.5%) is greatly increased compared to the normal population (6.8%) (Stratton and Wooster, 1996; Whittemore et al., 1997). Sufferers of Li-Fraumeni syndrome, which predisposes to a variety of cancers, carry a germline mutation in the p53 tumour suppressor (Velculescu and El-Deiry, 1996). Other 'class II' or non-classical tumour suppressors affect cell phenotype by changes in their expression level. These include maspin (Sheng et al., 1996), the gap junction proteins connexin 26 and connexin 43 (Hirschi et al., 1996), and BRCA1 in non-familial breast cancer (Russell et al., 2000; Thompson et al., 1995; Wilson et al., 1999).

A subset of *ets* genes has also been implicated in inhibition of tumourigenesis. These include *Erf, PU.1, PSE, Elf5* and possibly *Ets-1, Ets-2* and *PEA3*. Over-expression of the haematopoietic *ets* factor, PU.1, in K562 myeloid leukaemia cells caused both reduced growth and clonogenicity, and increased monocytic differentiation in this cell line (Delgado et al., 1998). *Erf* suppresses the transformation of NIH3T3 cells by various oncogenes including the v-*ets* containing E26 (Sgouras et al., 1995), probably by antagonising its binding to cellular DNA targets. *PSE* and *ELF5* are discussed in Section 1.6.

There are conflicting reports in the literature with *ets* genes such as *PEA3* and *Ets-2* as to whether their role in cancer is as a growth-enhancer or, indeed, as a repressor. PEA3

transrepresses the promoter of the oncogene *HER-2/neu/ErbB2* and in various breast-derived carcinoma cell lines, mediates apoptosis, inhibits anchorage independent growth, and inhibits tumour formation in nude mice (Guerra-Vladusic et al., 2001; Shepherd et al., 2001; Xing et al., 2000). *PEA3* subfamily members have been detected in benign breast epithelium and in estrogen receptor positive breast tumours, but not in receptor-negative cells (Baert et al., 1997), while its expression appears to be absent in prostate cancer (Gavrilov et al., 2001). Conversely, other studies indicate that *PEA3* acts as an oncogene in the breast and other organs (Section 1.5.1). Differences in study design, origin of the cancers studied and comparisons between experiments performed in *in vitro* and *in vivo* contexts may contribute to these controversial reports, and a final conclusion is not yet possible.

Several older *ets-2* studies associate over-expression of *ets-2,* and genomic alterations at its locus, with immortalised or carcinoma cell lines and leukaemias (Dreyfus et al., 1989; Le Beau et al., 1986; Sacchi et al., 1986; Simpson et al., 1997). Furthermore, expression of antisense *ets-2* in hepatoma cells inhibited growth, and reduced the capability of the cells to grow in an anchorage independent format and to form tumours in nude mice (Huang et al., 1994). More recent studies suggest that Ets-2 promotes apoptosis and requires p53 to translocate to the nucleus to have this effect (Sanij et al., 2001; Wolvetang et al., 2003). Therefore, in p53-null cells Ets-2 may not be capable of acting as a tumour suppressor, though in physiologically normal cells this is its function.

Likewise, while *Ets-1* was originally associated with leukaemia and lymphoma and even with potentially invasive phenotypes in oesophageal cancer (Saeki et al., 2002), the short p42 isoform has been shown to rescue defects in Fas-induced apoptosis in a colon cancer cell line. Its ability to activate apoptosis by upregulating interleukin 1β-converting enzyme (ICE)/caspase 1, where normal apoptotic pathways are blocked, indicates that it has tumour suppressor capability (Li et al., 1999). Its dual functions as an oncogene/tumour suppressor gene seems to therefore result from different activites of alternative splice isoforms, or to depend on the cellular status of other cancer-related genes, as described above for *Ets-2*. Recently, epithelial-specific *ets* factors (ESEs) have been described as candidate oncogenes and tumour suppressor genes (Section 1.6).

1.6 Studying breast epithelial cancer

Epithelial cancers are a clinically relevant, common class of solid tumours, particularly in adult humans. About 85% of cancers are carcinomas or adenomas derived from aberrant gene expression in mainly glandular epithelial tissues. Breast cancer, in particular, is a major cause of morbidity and accounts for 18% of all malignancies in women (Haimov-Kochman et al., 2002). Studying the molecular mechanisms by which epithelial carcinomas arise will contribute to understanding of normal development and tumourigenesis, leading eventually to the development of more specific and effective treatments.

In breast carcinoma, the mechanisms linking normal development and tumourigenesis are particularly likely to overlap significantly. As the breast is constantly being remodeled during the reproductive lifetime in females, cells must retain the potential to proliferate and differentiate – a property taken advantage of by cancerous cells. During pregnancy, mammary epithelium proliferates and terminally differentiates into milk-secreting alveoli, and the fat cells de-differentiate into small pre-adipocytes (Hennighausen and Robinson, 2001). Post-lactation, the mammary gland involutes, the alveolar epithelium regresses by apoptosis and the fat cells re-differentiate until the gland resembles its previous state (Alexander et al., 2001).

Normal mammary epithelial cells therefore retain many cancer-like properties. These include invasion, which occurs during the proliferation of the epithelial ductal structures at puberty; re-initiation of cell proliferation, which starts again in each pregnancy; resistance to apoptosis, by mechanisms that prevent premature involution; and angiogenic remodeling, which must take place to service the changing architecture of the gland (reviewed in Wiseman and Werb, 2002). Therefore, it is predictable that many factors, both cell-autonomous and stromal factors, involved in mammary gland development are also associated with mammary carcinogenesis.

For example, inactivation of the TGFβ signalling pathway, frequently by defects in TGFβRII expression, contributes to breast and gastric carcinogenesis. The epithelial-specific *ets* factor, *ELF3*, is a critical regulator of the tumour suppressor *TGFβRII* (Choi et al., 1998) and is required for its expression in the enterocytes of the small intestine (Ng et

al., 2002). *ELF3* modulates squamous differentiation-associated genes such as *keratin-4*, *SPRR2A*, *profillagrin*, *keratin 8* and *TGM3* in epithelial cells (Andreoli et al., 1997; Brembeck et al., 2000; Choi et al., 1998; Oettgen et al., 1997b). Loss of ELF3 protein may result in TGFβRII deficiency in gastric cancer. Given the involvement of *ELF3* with mammary gland development and involution (Neve et al., 1998) and the presence of TGFβRII in breast tissue, this mechanism may be predicted to hold true for mammary carcinogenesis also.

Recently, *ELF3* status and activity in breast cancer has been studied more extensively, contradicting predictions that it is associated with tumour suppression in this organ. Amplification of the 1q32 locus that encompasses *ELF3* has been identified in HER2/*neu* amplified cell lines (Chang, 1997) and in about 50% of early breast tumours (Isola et al., 1995), associating increases in *ELF3* transcript levels with mammary carcinogenesis. *ELF3* has been shown to stimulate the HER2/*neu* and collagenase (MMP1) promoters (Eckel et al., 2003), indicating that a positive feedback loop may exist between *ELF3* and HER2/*neu*. Ectopic expression of *ELF3* (or of *Ets-2*) in immortalised, *ELF3* negative MCF-12A cells conferred colony-forming ability on these cells while expression of dominant-negative *ELF3* in *ELF3* positive T47D breast cancer cells inhibited colony formation (Eckel et al., 2003).

Several other *ets* transcription factors have been implicated in mammary gland development and carcinogenesis. These include *Pea3, Elf5, PSE* and *ESE3*. Ubiquitously expressed *ets* genes such as *GABP* and *Ets-2* and *Ets-1* are also expressed in mammary

epithelium, and *GABP* activity has also been linked to breast carcinogenesis, as it is required for *BRCA1* promoter activity (Atlas et al., 2000). It may also play an indirect role in mammary gland development during pregnancy, as it appears to mediate transcription of the lactogenic hormone prolactin in response to insulin (Ouyang et al., 1996). *Ets-1* but not *Ets-2* has been shown to be critical for the expression of prolactin in decidua via one of six *ets* motifs in the decidual-type promoter, not utilised in the pituitary. This may also be relevant to the expression of prolactin in mammary epithelial proliferation and differentiation in pregnancy (Bradford et al., 2000; Brar et al., 2002; Schweppe and Gutierrez-Hartmann, 2001).

Recently, over-expression of the prostate-derived *ets* transcript *PSE* has been identified in 14/20 breast tumours (Ghadersohi and Sood, 2001) and in breast and prostate cancer libraries (Mitas et al., 2002). The gene is expressed in normal breast as well as prostate epithelium (Feldman et al., 2003a; Nozawa et al., 2000). Using quantitative RT-PCR, expression of *PSE* was >100-fold higher compared with matched normal samples, which barely showed detectable expression. In one breast cancer patient, *PSE* transcript levels were discovered in blood at 192-fold greater levels than in two normal and one other breast cancer patient (Ghadersohi and Sood, 2001). These data suggest that *PSE* may be a candidate breast cancer marker and antigen. However, no data are available to suggest a reason for the inappropriate expression of *PSE* in breast cancer, nor any advantage the presence of *PSE* mRNA may provide for tumourigenic cells. Subsequent studies focused on the protein, rather than mRNA, expression of *PSE* in breast cancer. These showed reduction of protein levels in invasive breast cancer and absence in invasive breast cancer

cell lines. Re-expression of *PSE* in breast cancer cell lines led to abrogation of the growth, migration and invasion capabilities of the cells, partly owing to G_0-G_1 cell cycle arrest resulting from increased p21 levels. These data indicate that *PSE* is a tumour suppressor gene, and that the loss of PSE protein in breast epithelium allows breast cancer progression and invasion by altering the expression of a spectrum of regulatory genes (Feldman et al., 2003a).

Expression of *ets* genes has been implicated in epithelial-mesenchymal interactions and in regulation of ECM proteins and enzymes that cleave ECM components, such as MMPs. Due to their proteolytic activity, MMPs have important roles in mammary gland remodeling in both normal and cancerous states. Their activity is particularly relevant in the continuously remodeling mammary gland tissue. Stromal expression of MMPs has been associated with metastatic phenotypes of human ductal carcinoma. MMP-mediated cleavage of the ECM component, laminin-5, releases active peptides, which are necessary for mammary epithelial migration, but in cancer may be an inducer of metastasis (Koshikawa et al., 2000). Mice carrying targeted deletions of MMP-3 show reduced side branching of the epithelial tree (Sternlicht et al., 1999). Both MMPs and tissue inhibitors of metalloproteinases (TIMPs) are regulated by *ets* genes such as *Ets-1* (Gomez et al., 1997; Grevin et al., 1993; Thompson et al., 1992), *Ets-2* (Yamamoto et al., 1998) and the PEA3 subfamily. Additionally, *Ets*-1 is expressed in the invading terminal end buds in the later developmental stages of the gland. Its expression correlates with angiogenesis and stromal proliferation in tumourigenesis (Delannoy-Courdent et al., 1998). The three genes that comprise the PEA3 subfamily have overlapping expression patterns that reflect their

function in epithelial-mesenchymal interaction (Chotteau-Lelievre et al., 1997). They affect morphogenesis by transcriptionally regulating MMPs in mammary gland development, migration and invasion (Higashino et al., 1995; Kaya et al., 1996; Matrisian, 1992; Wasylyk et al., 1991). They may also have this function in malignant mammary tissue, as PEA3 is over-expressed in 76% of tumours, and in almost all ErbB2-positive tumours (Benz et al., 1997; Chotteau-Lelievre et al., 1997; Trimble et al., 1993; Xing et al., 2000).

No work has been published to date on whether these remodeling and ECM proteins are also transcriptionally regulated by ESEs. Speculatively, it is likely that ESEs do transcriptionally regulate these genes given that even in the ESE subfamily, the *ets* domain is highly conserved and is capable of binding the EBS in the promoters of MMPs, TIMPs and other relevant genes. Additionally, many *ets* target genes are known to be co-ordinately regulated by multiple *ets* proteins, rather than by a single member of the family (Bemark et al., 1999; Garret-Sinha et al., 2001; Hu et al., 2001; Kas et al., 2000). ELF3, ELF5 and ESE-3 are all expressed in mammary epithelium (Kas et al., 2000; Tymms et al., 1997; Zhou et al., 1998).

1.7 ELF5, an epithelial-specific ets (ESE) transcription factor

This study addresses the relationship of the epithelial-specific *ets* gene, *ELF5*, to mechanisms of mammary carcinogenesis. *ELF5* activity is crucial for the development of

the mammary gland throughout the reproductive cycle, and circumstantial evidence is consistent with the hypothesis that *ELF5* may be a tumour suppressor gene.

ELF5 was first published as an epithelial-specific *ets* factor in 1998, based on homology within its DNA-binding domain to the ELF/E74 subfamily of *ets* proteins. The relatively divergent *ets* domain is most similar to that of *ELF3,* with 67% amino acid identity (Zhou et al., 1998). It is also 65% similar to the recently discovered ESE-3, indicating that these three proteins form a new, separate subfamily of *ets* genes (Kas et al., 2000). *ELF5* also possesses an N-terminal *pointed* protein interaction domain. Outside these regions, there is little similarity to other *ets* factors. *ELF5* acts as a transcription factor, displaying *ets*-like sequence-specific DNA binding characteristics. Unlike the prototype family members *Ets-1* and *Ets-2*, *ELF5* does not possess the MAPK site involved in enhancing transcriptional activity, located at the N-terminal end of the *pointed* domain. However, this does not preclude it from MAPK regulation, as the closely related ESE-3 is a MAPK target, probably via a site in the central region of the protein (Kas et al., 2000; Tugores et al., 2001).

Elf5 mRNA is expressed in a highly restricted manner in organs rich in glandular epithelium, and also in differentiated keratinocytes. Expression is highest in salivary gland, followed by mammary gland, the respiratory tract, kidney, stomach and prostate (Oettgen et al., 1999b; Zhou et al., 1998). This pattern is restricted compared to *Elf3* and *Ese-3,* which are expressed widely in epithelial-rich organs including the gastrointestinal tract, pancreas and ovary, as well as those in which *Elf5* is found. This indicates a role for *Elf5* in the

regulation of glandular epithelial cell gene regulation and differentiation. *Ets* binding sites from the epithelial-expressed genes *WAP* (Thomas et al., 2000), *CRISP-1, CRISP-3, PSP, PSA, MP6, SPRR2A* and *keratin 18/EndoA* (Oettgen et al., 1999b) have been shown to be bound by Elf5 protein *in vitro*. These genes are therefore potential targets for regulation by *Elf5 in vivo*.

New evidence shows that *ELF5* is required for development of the lactating mammary gland in the mouse. Female mice carrying a heterozygous deletion of *Elf5* fail to lactate, so that pups die as neonates (Appendix VII). This phenotype is similar, if more severe, to that found in the prolactin receptor (*PRLR)* heterozygous mice (Lucas et al., 1998) and the *Stat5a* knockout line (Liu et al., 1998). Notably, *Stat5* co-immunoprecipitates with *Ets-2* in stimulated T cells, demonstrating that it can form a transcriptional activation complex with *ets* proteins (Rameil et al., 2000).

The hypothesis that *ELF5* acts as an epithelial-specific tumour suppressor is based upon several pieces of evidence. Firstly, the human *ELF5* locus is situated on chromosome 11p13, a region that harbours both defined and undefined tumour suppressive elements (Goldberg et al., 2000; Harris et al., 1991; Lee and Haber, 2001). It is a part of the genome that commonly undergoes LOH in a variety of epithelial cell carcinomas. In particular, deletions involving regions on 11p13 have been found in solid tumours of different origins including breast (Fabre et al., 1999; Gudmundsson et al., 1995; Karnik et al., 1998b; Winqvist et al., 1995), lung (Bepler et al., 1998; Ludwig et al., 1991; Shipman et al., 1998; Tran and Newsham, 1996; Virmani et al., 1998), ovarian (Vandamme et al., 1992; Viel et

al., 1992), bladder (Koo et al., 1999; Shaw and Knowles, 1995; Shipman et al., 1993), melanoma (Goldberg et al., 2000) and stomach (Baffa et al., 1996) carcinomas, and in childhood cancers including the kidney-derived Wilms' tumour (Karnik et al., 1998a; Koufos et al., 1989; Nakadate et al., 2001; Reeve et al., 1989), hepatoblastoma (Albrecht et al., 1994) and rhabdomyosarcoma (Besnard-Guerin et al., 1996; Visser et al., 1997). If *ELF5* is a locus encompassed by these cancer-specific deletions, this may indicate that loss of *ELF5* is a step necessary for the development of carcinoma. While none of these studies examined *ELF5* specifically, they did examine genes closely linked to the *ELF5* locus, such as *catalase* (*CAT*) which is situated only 7 kilobases (kb) 3′ to *ELF5,* and *Wilms' tumour* (*WT1*) (Ludwig et al., 1991; Shipman et al., 1993). Deletions of these genes are likely to also encompass *ELF5*. Heritable developmental syndromes such as Beckwith-Wiedemann syndrome are associated with deletions of the 11p13-15 region. One phenotype of this syndrome is predisposition toward childhood cancer, particularly kidney-derived tumours. *ELF5,* in addition to the *WT-1* locus, which is not deleted in all Wilms' tumours, may therefore be a candidate gene for involvement with this phenotype.

Secondly, *ELF5* mRNA expression in a variety of epithelial-derived cancer cell lines is lost, compared to its expression in the organs from which the cell lines are derived (Oettgen et al., 1999b; Zhou et al., 1998). The cell lines tested derive from breast, kidney, lung, prostate and endometrium, and all but two do not express the mRNA. The two cell lines retaining expression are the steroid hormone-responsive, non-metastatic lines T47D (breast carcinoma) and LNCaP (prostate carcinoma). This may indicate that loss of *ELF5* mRNA is associated with conversion to a more aggressive phenotype. Evidence for loss or

rearrangement of an *ELF5* allele, as evaluated by Southern blot analysis, was found in four of the nine lung carcinoma cell lines tested. T47D cells may have amplification of the *ELF5* locus, while another non-expressing cell line appeared to have both amplified and rearranged alleles (Zhou et al., 1998). A recent publication investigating gene expression profiles in human breast cancer by microarray and quantitative RT-PCR (QRT-PCR) analysis made particular note of the frequency and consistency of the downregulation of *ELF5* transcript in breast cancer. These results were so consistent as to be used as quality control measure in comparing breast cancer microarray data with single-gene QRT-PCR validation. In seven of eight atypical ductal hyperplasias (ADH), 28 of 30 ductal carcinoma *in situ* cases (DCIS) and 25 of 25 invasive ductal carcinomas, >2-fold under-expression of *ELF5* mRNA was noted by quantitative RT-PCR (Ma et al., 2003a). It is notable that with the increasing grade of the cancer, higher proportions of the samples downregulate *ELF5* expression. These data from primary breast samples provide convincing evidence to support the cell line data.

Interestingly, a second *ets* gene, *ESE-3,* is closely linked to *ELF5* on 11p12, orientated in a head-to-head direction, ~110 kb apart. *ESE-3* is most closely related to *ELF5* among the *ets* family, indicating that these genes arose from a duplication event. 11p12 also undergoes LOH in a variety of epithelial cancers and is a 'hot spot' for inherited deletion- or amplification-associated developmental disorders. The expression pattern of *ESE-3* is less restricted than for *ELF5*, with expression in the pancreas, colon and small intestine, appendix, ovary, testis, liver and pituitary, adrenal and thyroid glands in addition to the organs in which *ELF5* is expressed (Kleinbaum et al., 1999). ESE-3 immunoreactivity in

bladder, oral epithelial and breast carcinomas was lost compared to normal cells (Tugores et al., 2001). LOH at the 11p12-13 locus was not tested in these studies so it remains to be elucidated whether this gene is deleted, possibly along with linked genes such as *ELF5*, or inactivated by another mechanism. However, *Elf5* and *Ese-3* must have different functions *in vivo,* as targeted deletion of *Ese-3* coding sequence in mice did not result in an obvious phenotype (Wasylyk et al., i998). In contrast, homozygous deletion of *Elf5* results in embryonic death in the peri-implantation window, and heterozygous deletion in developmental failure of the mammary gland at lactation (Appendix VII).

1.8 Aims

This study aimed to further the study of *ELF5* in normal development and in carcinogenesis by characterising the protein expression pattern of murine *ELF5* and by investigating the transcriptional regulation of the gene. Potential downstream target genes that are known to have oncogenic or tumour suppressor activity will be identified to examine if they are regulated by *ELF5*, as any role *ELF5* has in cancer will be intrinsically linked to its function as a transcription factor. The expression of *ELF5* in carcinomas derived from the organs it is normally expressed in will be examined, and functional studies in carcinoma cell lines performed to determine whether *ELF5* functions as a tumour suppressor gene in these cancer types.

Chapter 2. Methods

Gene Nomenclature

When referring to a gene name or product, the name is italicised for the gene and gene transcript, but not for the protein product. Human genes are capitalised, while the murine equivalent has only the first letter capitalised, and the remainder in lower-case lettering. Drosophila genes are referred to in lower-case lettering.

2.1 List of solutions, suppliers, equipment, sequences and oligonucleotides

Appendix I List of oligonucleotides

Appendix II List of solutions

Appendix III List of suppliers

Appendix IV List of equipment

Appendix V Output from transcription factor binding site searches

Appendix VI List of commonly used abbreviations

Appendix VII Zhou et. al., (2003); submitted to EMBO J

Unless otherwise specified, all centrifugation steps were carried out in a Sigma 1-15 benchtop microcentrifuge (Quantum Scientific).

2.2 DNA manipulations

2.2.1 Polymerase chain reaction (PCR)

2.2.1.1 Oligonucleotide design

Oligonucleotides were usually 18-24-mers, containing a similar number of guanine/cytosine and adenine/thymine base pairs and where possible, no stretches longer than three identical bases. A list of all the oligonucleotides used is presented in Appendix E.

2.2.1.2 Amplification Protocol

Unless otherwise specified, PCR reactions were performed in a total volume of 25 µl, using 0.2 units of Taq DNA polymerase in 1 x Taq DNA polymerase Thermo buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide (dATP, dTTP, dCTP, dGTP) (Promega), with 10 pmoles of each oligonucleotide primer (Proligo). The amount of template DNA added was approximately 1-10 ng for cDNA or plasmid DNA, and 100 ng for genomic DNA. Negative controls contained no template DNA.

When longer or high-fidelity PCR products were expected, Elongase Enzyme Mix (Invitrogen), Platinum Pfx (Invitrogen) or Pfu (GibcoBrl) DNA polymerases were used with their own buffers, instead of the usual Taq DNA polymerase, buffer and $MgCl_2$.

The PCR programs used contained an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles each of denaturation (94°C for 30 seconds), annealing (x°C for 30 seconds) and extension (74°C for y minutes), and a final extension step at 74°C for 5

minutes. An extension temperature of 68°C was used for Platinum Pfx and Elongase enzymes.

x°C = T_m - 5 where T_m = 2(A+T) + 4(G+C) = melting temperature of primer.

y minutes = 1 minute/kb of PCR product (minimum 20 seconds).

For the *normal* program, annealing was performed for all cycles at the same temperature. For the *touchdown* program, annealing temperature was decreased by 1°C for each of the first 10 cycles, and was then kept constant for the remaining cycles.

PCR products were analysed by agarose gel electrophoresis, as described in Section 2.4. Where required, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), or excised from agarose gels, as described in Section 2.2.4.2.

2.2.2 DNA cloning

2.2.2.1 Restriction endonuclease digests

Restriction endonuclease digests were performed using Promega reagents. They contained DNA (PCR products (ng), plasmid DNA (ng-µg) or genomic DNA (20 µg), 1-10 U of the appropriate restriction endonuclease per µg DNA, 1 X restriction enzyme buffer (Promega) and distilled H_2O. The reaction was incubated at the appropriate temperature for 2 hours for plasmid DNA to overnight for genomic DNA digests, and analysed by agarose gel electrophoresis.

2.2.2.2 Blunt-ending DNA fragments

The termini of restriction endonuclease digested DNA fragments and vector DNA that had non-compatible cohesive termini were rendered blunt-ended before ligation. Klenow DNA polymerase fill-in reactions were performed using Promega Klenow DNA polymerase and buffer. DNA with a 5′ overhang was incubated with Klenow DNA polymerase and 1 X buffer, 100 µM dNTPs and the digested DNA in a 100 µl volume. DNA with a 3′ overhang was supplemented with 25 U Klenow DNA polymerase, 1 X buffer and the DNA in a 100 µl volume. Each mixture was incubated at room temperature for 15 minutes then at 65°C for 15 minutes to stop the reaction. Excess deoxynucleotides were removed by phenol-chloroform extraction and ethanol precipitation.

2.2.2.3 Removal of 5'-phosphate moieties

To avoid vector recircularisation during ligation, the restriction endonuclease linearised vector DNA was dephosphorylated by incubation with 0.1 U calf intestinal alkaline phosphatase (CIAP) and 1 X buffer (Roche) and the digested DNA in volume of 100 µl. For DNA with 5′ overhangs, the mixture was incubated at 37°C for 30 minutes, then another 0.1 U of CIAP was added and the incubation continued for 30 minutes. For 3′ overhangs and blunt ends the mixture was incubated at 37°C for 15 minutes, then 56°C for 15 minutes. Another 0.1 U of CIAP was added for further incubation at 37°C and 56°C. The reaction was stopped by addition of 2 µl of 0.5 M EDTA and incubation at 65°C for 15 minutes. Proteins were removed from the DNA by phenol chloroform extraction and ethanol precipitation, and the DNA was quantified by agarose gel electrophoresis (Section 2.2.3).

2.2.2.4 Ligation

The amount of vector and insert DNA used for ligation was calculated using the formula:

$$\frac{\text{vector (ng)} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} \times (\text{molar ratio of insert:vector}) = \text{insert required (ng)}$$

Ligation reactions using 1:1 and 1:3 vector:insert ratios contained 50 ng of dephosphorylated vector, the appropriate amount of insert DNA, 1 U T4 DNA Ligase (Promega), 1 X T4 Ligase buffer in 10 µl. The ligation was incubated at approximately 15°C overnight. Control reactions containing dephosphorylated vector only, with and without T4 DNA ligase, were also set up to provide estimates of ligation efficiency.

2.2.2.5 Direct cloning of PCR products using the pGEM-T or pGEM-T Easy vectors

PCR products amplified with Taq DNA polymerase, which contain termini with protruding 3'-adenosine residues, were directly ligated to 50 ng pGEM-T or pGEM-T Easy vector (which contain complementary 3'-thymidine protruding termini; Promega). Blunt-ended PCR products generated with the Pfx DNA polymerase were cloned into pGEM-T or pGEM-T Easy after the enzymatic addition of a 3'-adenosine residue. To do so, the PCR product was first purified by phenol:chloroform extraction. The purified product was then incubated at 72°C for 15 minutes with 0.2 U of Taq DNA polymerase in 1 X Taq DNA polymerase Thermo buffer, 1.5 mM $MgCl_2$, and 200 µM of dATP (Promega).

2.2.2.6 Transformation of plasmid DNA into bacterial cells

Chemically competent *Escherischia coli (E. coli)* JM109 bacterial cells (Promega) were used to amplify plasmid DNA for subsequent purification (Section 2.2.5). For

expression and purification of recombinant proteins (Section 2.4.2), BL21(DE3)pLysS bacterial cells (Novagen) were used. Bacterial cells were thawed on ice then added to 10-20 ng of DNA and incubated on ice for 30 minutes to allow the DNA to enter the bacteria. The tube was then heated at 42°C for 45 seconds to heat shock the bacteria and seal the cell membrane. Immediately, the tube was quenched on ice for 2 minutes and 300 µl SOC medium was added. The mixture was incubated at 37°C for 45 minutes in a shaking incubator (Innova 4300, New Brunswick Scientific). Each transformation was plated onto a selective plate of Luria-Bertani (LB) agar including the appropriate antibiotics. When using the pGEM-T or pGEM-T Easy vectors, blue/white colour selection was used to identify putative recombinant bacteria by adding 20 µl 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Promega) and 20 µl 100 mM isopropylthio-β-D-galactosidase (IPTG; Promega) to the transformation reaction before plating.

2.2.2.7 Screening of ligation products

Screening by PCR

From each ligation and transformation, a number of colonies were chosen for analysis (white colonies only in the case of blue/white selection). Each colony was picked using a sterile 200 µl tip, which was then dipped into 50 µl TE pH 8.0, and also used to inoculate 100 µl LB medium containing the appropriate antibiotic. One µl of the bacteria-containing TE was used as a template for PCR to determine the presence of an insert, while the LB inoculum grew at 37°C pending the PCR analysis.

Screening by DNA digest

Alternatively, if the presence of the desired DNA could not be easily detected by PCR, plasmid clones could be prepared from individual bacterial colonies and analysed by DNA restriction endonuclease digestion and agarose gel electrophoresis. From the transformation plate, a number of colonies were chosen using a sterile tip. This was used to inoculate a master LB agar plate, to keep a record of the colonies, and then subsequently to inoculate 1 ml LB broth including the appropriate antibiotics. The broth was grown overnight with shaking at 37°C and the DNA prepared from the bacteria clone using a miniprep method (Section 2.2.5.1). The master plate was stored at 4°C and used for further inoculations.

2.2.3 DNA gel electrophoresis

Agarose gels of varying concentrations (0.8 % (w/v) to 2.0 % (w/v)) were prepared in 1 X TAE buffer using nuclease-free powdered agarose (Promega). DNA samples in 1 X DNA loading dye were loaded into wells and electrophoresed at 60-100 Volts (V) in 1 X TAE running buffer supplemented with approximately 100 µl ethidium bromide (1 mg/ml stock; Promega). Lambda phage DNA digested with *Eco*RI and *Hind*III, or *Hind*III alone was used as a DNA size marker. DNA was visualised by placing the gel on a short-wave ultraviolet (UV) transilluminator (Bio-Rad) and printed on thermal paper (Mitsubishi K61S-CE standard type, Mitsubishi) using the GelDoc 1000 system (Bio-Rad) with a P90E video copy processor (Mitsubishi).

2.2.4 DNA purification

2.2.4.1 Phenol chloroform extraction

To remove contaminating protein from DNA in solution, an equal amount of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA (minimum volume of 100 µl), and the mixture was vortexed and centrifuged at 14,000 rpm for 2 minutes. This step was repeated until no protein precipitate was visible at the interphase. The upper phase was collected and the DNA precipitated by addition of 100 µg glycogen, 1/10 volume 7.5 M ammonium acetate (NH_4OAc), and 2.5-3 volumes 100 % (v/v) ethanol, and then pelleted by centrifugation at 14,000 rpm for 10 minutes. The DNA pellet was washed with 70 % (v/v) ethanol, air-dried and resuspended in autoclaved distilled water (dH_2O) or TE (pH 8.0) buffer.

2.2.4.2 Agarose gel purification

DNA generated by PCR or by restriction endonuclease digestion of plasmid, was excised using a scalpel blade from agarose gels under short-wave UV light, and purified using the Qiaex II Gel Extraction Kit, according to the manufacturer's instructions (Qiagen).

2.2.5 DNA isolation from bacterial cells

2.1.5.1 Small scale DNA isolation miniprep

Bacterial cultures were grown for up to 16 hours, at 37°C with shaking, in LB broth including the appropriate antibiotic. The cultures were pelleted by centrifugation at 14,000 rpm, resuspended in Solution 1 and lysed with Solution II (0.2 N NaOH/1 % SDS). After incubation on ice for 5 minutes, the solution was neutralised with Solution III (5 M potassium acetate (KOAc) (pH 4.8)) and chilled on ice for 10 minutes. Cellular

debris was pelleted in a microcentrifuge for 10 minutes. Ribonuclease A (RNase A) (Roche) was added to the supernatant at a concentration of 50 µg/ml and incubated at 37°C for 30 minutes. The solution was extracted with chloroform:isoamyl alcohol (24:1) until no proteins were visible at the interphase. DNA was precipitated from the aqueous phase with 2 volumes of ethanol, washed with 70% ethanol and resuspended in TE (pH 8.0).

2.2.5.2 Large scale DNA isolation maxiprep

Large quantities of highly pure plasmid DNA required for transfection into mammalian cells were prepared using the Qiagen Endo-Free Maxiprep kit according to the manufacturer's instructions, and quantified at OD_{260nm} and OD_{280nm}. DNA concentration was calculated according to the formula:

DNA concentration (mg/ml) = OD_{260nm} x 50 mg/ml x dilution factor

2.2.6 DNA isolation from mammalian cells

Cells ($1x10^5$-$1x10^6$ cells) were lysed in 5 ml cell lysis buffer and added to 50 µl of 10 mg/ml Proteinase K (Roche) to digest cellular proteins. The lysed cells were incubated at 55°C for 4 hours. Genomic DNA was precipitated by addition of 2 volumes of 100 % (v/v) isopropyl alcohol and spooled out onto a sealed glass pipette. DNA adhered to the pipette and was washed twice in 80 % (v/v) ethanol before being air-dried and resuspended in 200 µl TE (pH 8.0). If required, DNA was further purified by phenol-chloroform extraction (Section 2.2.4.1).

2.2.7 DNA isolation from peripheral blood lymphocytes

Whole blood (one ml) was mixed with 12 ml cold dH_2O and incubated on ice for 10 min. Nuclei were pelleted by centrifugation at 2000g (Biofuge Stratos) for 10 min at 4°C. Nuclei were resuspended in 12 ml cold 0.1 % (v/v) Nonidet-P40, incubated on ice for 10 min and centrifuged as above. Nuclei were lysed by addition of 5 ml cold blood lysing solution, 250 µl 10 % (w/v) SDS and 0.5 mg Proteinase K, and incubated for 4 hours at 37°C. Genomic DNA was separated from other cellular components by addition of 1/5 volume 5 X ANE and two phenol:chloroform extractions (Section 2.2.4.1), followed by one chloroform:isoamyl alcohol (24:1) extraction. DNA was precipitated from the aqueous phase by addition of 1/10 volume of 4 M NaCl and 2 volumes of 100 % (v/v) ethanol. The precipitated DNA was pelleted by centrifugation at 2000g (Biofuge Stratos) for 15 min, rinsed in 80 % (v/v) ethanol and air-dried. DNA was re-dissolved in 300µl TE (pH 8.0) buffer at 55°C overnight and stored at 4°C.

2.2.8 DNA sequencing analysis

Sequencing reactions and gels were performed by the Wellcome Trust Sequencing Centre, a joint facility of Monash Institute of Reproduction and Development and Prince Henry's Institute of Medical Research, using the ABI-PRISM BigDye Terminator chemistry (Version 3.4.1), on an Applied Biosystems Model 373A automatic DNA sequencer.

2.2.9 DNA radioactive hybridisation

2.2.9.1 Random-primed labelling of double-stranded DNA

Double-stranded DNA probes were labelled with [α-^{32}P] dCTP (10 mCi/ml; Amersham-Pharmacia) using a random-primed DNA labelling kit, according to the manufacturer's instructions (Roche). Unincorporated [α-^{32}P] dCTP was removed by column chromatography using a 2 ml Poly-Prep Chromatography column (Bio-Rad) containing G-50 Sephadex beads (Amersham Pharmacia) in TE (pH 8.0). The specific activity of the labelled DNA was determined using a β-scintillation counter (Wallace Scintillation Counter) as counts per minute (cpm) in the presence of 5 ml hydrofluor scintillation fluid (BDH Biochemicals). The labelled DNA was then denatured by heating at 95°C for 5 minutes, and added to the pre-hybridisation solution to obtain a final concentration between 0.1 and 10 ng of probe/ml.

2.2.9.2 End-labelling of single-stranded oligonucleotide primers

Single-stranded oligonucleotide primers were radiolabelled with [γ-^{32}P] dATP or [γ-^{33}P] dATP (10 mCi/ml; Amersham-Pharmacia). In a 1.5 ml microfuge tube, 10 pmol DNA oligonucleotide primer was incubated in 1 X polynucleotide kinase buffer, with 10 U T4 polynucleotide kinase (Promega) and 2 µl [γ-^{32}P] dATP in a total volume of 10 µl. The mixture was left at 37°C for 30 minutes, before use for hybridisation.

2.2.9.3 Southern analysis

PCR products or restriction endonuclease-digested DNA was electrophoresed through an agarose gel and transferred to a Genescreen Plus membrane (NEN) by capillary action alkaline transfer according to the manufacturer's instructions. Freshly prepared 0.4 N

NaOH was used as the transfer buffer. The transfer was allowed to proceed overnight then the membrane was rinsed in 2 X SSC and air-dried prior to hybridisation.

For random-primed labelled DNA hybridisations, DNA membranes were pre-hybridised for at least one hour at 65°C in hybridisation solution, and hybridised overnight at 65°C with denatured [α-^{32}P] dCTP-labelled DNA and 1 mg denatured herring sperm DNA. After two 15 minute washes at 65°C with 2 X SSC containing 0.1 % (w/v) SDS, followed by one 15 minute wash with 0.1 X SSC containing 0.1 % (w/v) SDS, the membranes were exposed to a Fuji phosphorimage screen overnight and visualised using a phosphorimage analyser (FLA-2000, Fujifilm). Alternatively, membranes were exposed to BioMax MR X-ray film (Kodak) to visualise the specifically hybridised radioactivity.

For end-labelled oligonucleotide hybridisations, DNA membranes were pre-hybridised for one hour at 42°C in oligonucleotide hybridisation solution, and hybridised overnight at 42°C with the [γ-^{32}P] ATP-labelled oligonucleotide and 1 mg denatured herring sperm DNA. After two 15 minute washes at 42°C with 2 X SSC containing 0.1 % (w/v) SDS, the membranes were exposed to a Fuji phosphorimage screen for one hour to overnight, and visualised using a phosphorimage analyser. Alternatively, the membranes were exposed to BioMax MR X-ray film for at least 4 hours and developed for analysis.

Where required, the DNA membranes were stripped of residual radioactivity by incubation in 0.1 % (w/v) SDS preheated to 90°C, with gentle shaking until the solution cooled to room temperature.

2.3 RNA manipulations

All RNA solutions were treated with 0.1 % (v/v) diethyl pyrocarbonate (DEPC; Sigma) overnight and autoclaved prior to use.

2.3.1 PolyA$^+$ RNA extraction from cultured cells or tissue

2.3.1.1 Oligo-dT preparation

One gram of oligo-dT cellulose (Boehringer-Mannheim) was incubated with 20 ml 0.4 M NaOH for 5 minutes before centrifugation at 1000 rpm (Biofuge Stratos) for 5 minutes at 4°C. The oligo-dT pellet was resuspended in 20 ml 1 M Tris pH 7.5, and after a 5 minute incubation was spun again. The pellet was then washed in binding buffer, spun again and finally resuspended in binding buffer. The prepared oligo-dT solution was stored at 4°C.

2.3.1.2 RNA extraction

PolyA$^+$ RNA was extracted from tissues or cells using oligo dT cellulose. The snap-frozen tissues or cell pellets were homogenised (Ika Ultra Turrax T25) for 2 minutes in 25 ml of ice-cold RNA extraction buffer and 10 mg Proteinase K (Roche). Following incubation at 55°C for 30 minutes, 2.5 ml 5 M NaCl and 2.5 ml of oligo-dT cellulose resuspended in RNA binding buffer were added. The mixture was rotated for 2 hours at room temperature to allow RNA binding to the oligo-dT cellulose. The RNA-bound oligo-dT cellulose was washed twice with 10 ml RNA binding buffer, and once with 10 ml RNA wash buffer; each wash step involved a centrifugation step at 1,500 rpm (Biofuge Stratos) for 3 minutes. The RNA was eluted by addition of 2 ml RNA elution

buffer at 60°C for five minutes, and the elution step was repeated with 1.5 ml RNA elution buffer. The two RNA eluates were combined, and extracted first with 3 ml phenol, followed by 3 ml chloroform. The RNA was precipitated by addition of 400 µl 3 M NaOAc (pH 4.0) and 8 ml 100 % (v/v) ethanol and incubated overnight at -20°C, then pelleted by centrifugation at 10,000 rpm (JA-20 rotor, Beckman) for 1 hour at 4°C. The RNA pellet was washed with 70 % (v/v) ethanol, air-dried then resuspended in 200 µl TE (pH 8.0) and 1 U ribonuclease inhibitor (RNasin; Promega). A ten µl aliquot of the sample was diluted with 400 µl TE (pH 8.0) for the determination of RNA concentration and quality by UV spectrophotometry (Lambda Bio 20, Perkin Elmer) at OD_{260nm}, where RNA concentration = OD_{260nm} x 40 x dilution factor = ng/µl. The remaining RNA was stored at -70°C in the presence of 1/10 volume of 3 M NaOAc (pH 4.0) and 2.5 volumes of 100 % (v/v) ethanol.

2.3.2 Preparation of total RNA from small numbers of cells

Total RNA was extracted from tissues or cells by acid-phenol guanidine thiocyanate extraction (Chomczynski and Sacchi, 1987). Snap frozen cell pellets were resuspended in 10 volumes of homogenisation buffer (1 ml/100 mg of tissue of ice-cold Solution D/β-mercaptoethanol (10 ml/72 µl) and homogenised at the maximum speed (Ika Ultra Turrax T25, Ika). One tenth of the total volume of cold 2 M NaOAc (pH 4.0), 1 volume phenol and 0.2 volumes ice-cold chloroform:isoamyl alcohol (24:1) were added, incubated on ice for 20 minutes and centrifuged at 5000 rpm for 20 minutes (Biofuge Stratos). The aqueous phase was removed and 1 volume phenol and 0.2 volumes chloroform:isoamyl alcohol added, and centrifuged as above. The aqueous phase was then added to 1 volume of isopropanol and 20 µg/ml glycogen and precipitated at -20°C

overnight, then centrifuged as above. The pellet was washed with 1 ml 75 % (v/v) ethanol and centrifuged at 13,000 rpm for 10 minutes at 4°C. The RNA pellet was air-dried, resuspended in 20-50 µl DEPC-treated H_2O and stored at -80°C.

2.3.3 Reverse Transcription PCR of poly-A^+ or total RNA

The Superscript II or Superscript III Reverse Transcription (RT) Kit (GibcoBRL) was used to perform first strand DNA synthesis for 1 µg total RNA or 500 ng polyA^+ RNA to be used in subsequent reverse transcription PCR (RT-PCR) applications. For each +RT reaction, an equivalent –RT reaction was performed, containing all components except the reverse transcriptase enzyme, allowing genomic DNA contamination to be identified by PCR. All reactions were carried out according to the manufacturer's specifications.

2.4 Protein manipulations

2.4.1 Protein extractions from mouse tissues or mammalian cells

Mouse tissues were collected and either snap-frozen in liquid nitrogen and stored at -80° C, or used immediately. Mammalian cells were trypsinised, washed in PBS and snap frozen, or used immediately. Tissues were homogenised in the presence of RIPA buffer (100 µl/100 mg of tissue) containing protease inhibitors (1 protease inhibitor cocktail tablet per 10ml buffer; Roche) in a microfuge tube using a 1.5ml glass pestle (Kontes Glass Company, Biolab Australia Ltd). Cells were resuspended and lysed by repeated passage through a 10 ml syringe using a 22 gauge needle, in RIPA buffer as above. An equal amount of 2 X EDTA sample buffer was then added to the homogenate, and the

suspension was boiled for 10 minutes before centrifugation at 13,000 rpm for 10 minutes at 4°C.

2.4.2 Recombinant protein preparation

Recombinant protein was made using the appropriate cDNA linked to a $(HIS)_6$ tag in either the PQE-30 (Qiagen) or a pET21 series (Clontech) vector. Transformed bacteria were grown overnight in LB medium containing 50 µg/ml ampicillin sulphate in a 25 ml volume at 37°C. The 25 ml starter culture was then diluted into 250 ml with fresh media containing antibiotic. The culture was grown to OD_{600}=0.8-1.0 before inducing protein synthesis with 1 mM IPTG for 3 hours. The culture was then centrifuged at 5000 rpm (Beckman JA10 rotor) for 10 minutes at room temperature. Supernatant was removed and the cells were resuspended in 10 ml guanidine lysis buffer, then rotated for 30 minutes at room temperature. Cells were sonicated (4710 Series Ultrasonics Homogeniser, Parmer Instrument Co.) for 30 seconds and centrifuged at 5000 rpm (Biofuge Stratos) for 20 minutes at room temperature. Samples of the pellet and supernatant were taken for gel electrophoresis. TALON beads (800 µl) (Clontech) were resuspended in 400 µl guanidine lysis buffer and added to the cell supernatant. This mixture was rotated for 30 minutes at room temperature, then the beads were pelleted and transferred to a 2 ml Poly-Prep Chromatography column (Bio-Rad). A sample of supernatant was taken for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Beads were washed with 15 ml guanidine lysis buffer followed by 15 ml renaturation buffer. The protein was eluted from the beads with 6 aliquots of 400 µl native protein elution buffer. Samples of fractions and remaining beads were taken for SDS-PAGE analysis.

2.4.3 One-dimensional polyacrylamide gel electrophoresis

Discontinous denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve proteins on a gel according to the method of Laemmli (Laemmli et al., 1970). The separation (running) layer of the gel (12 % (w/v) acrylamide for separation of Elf5 proteins) was poured into bulk lots of 1.5 mm mini-gels in a casting unit (Mighty Small multiple gel caster SE200, Hoefer Scientific Instruments, Amersham Pharmacia). The gels were overlaid with water-saturated butanol and allowed to polymerise at room temperature. The butanol layer was removed by gently running tap water over the casting unit. The 5 % (w/v) acrylamide stacking layer of the gel was then poured above the separation layer of the gel, a comb to form the wells inserted and the gel allowed to polymerise. The gel was placed into the electrophoresis unit (MightySmall II SE250, Hoefer Scientific Instruments, Amersham Pharmacia) and covered with 1 x SDS-PAGE running buffer. Samples were supplemented with an equal volume of 2 x EDTA sample buffer, boiled for 10 minutes and loaded. The Benchmark Pre-stained protein ladder (Gibco-BRL) was used as a size marker. Electrophoresis was undertaken at 80 V through the stacking gel and 100 V through the separation gel until the dye front reached the bottom of the gel. Proteins were then either visualised by staining with Coomassie Brilliant Blue (R-250) staining solution or electroblotted (Section 2.4.5).

2.4.4 SDS-polyacrylamide protein gel staining

SDS-PAGE fractionated proteins were visualised by first staining in a Coomassie Brilliant Blue R-250 (Bio-Rad) staining solution for 30 minutes, then destaining overnight in Coommassie Brilliant Blue destaining solution. The gels were then stored

between two layers of wet cellophane sheets clipped onto a glass plate, and allowed to dry overnight.

2.4.5 Western analysis

SDS-PAGE fractionated proteins were transferred by electroblotting onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore) using a Semi-Dry Transfer Unit (TE77 SemiPhor, Hoefer Scientific Instruments, Amersham Pharmacia). As recommended by the manufacturer, two layers of Whatmann 3MM paper soaked in anode buffer I were first laid on the transfer apparatus anode plate, followed by one layer of paper soaked in anode buffer II. The Immobilon-P membrane was treated for 15 seconds in 100 % (v/v) methanol, for two minutes in MilliQ water, and five minutes in anode buffer II, before being placed on the anode buffer II-soaked paper. The protein gel was briefly soaked in anode buffer II before being placed on top of the membrane. Finally, three layers of Whatmann 3MM paper soaked in cathode buffer were added, before addition of the cathode plate. The proteins were electroblotted at 15-20 V for 1 hour, after which the membrane was soaked in 100 % (v/v) methanol for 10 seconds, before air-drying for 20 minutes.

Protein membranes were blocked for two hours at room temperature in 10 % (w/v) skim milk powder in 1 X TBST with gentle shaking. The immobilised proteins were then probed with the appropriate dilution of the primary antibody in 5 % (w/v) skim milk powder in 1 X TBST overnight at 4°C. After washing four times for 15 minutes each in 1 X TBST, the membranes were incubated for one hour at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody in fresh 5% (w/v) skim milk powder in 1 X TBST. The membranes were then washed as described

above, before incubation with SuperSignal West Pico chemiluminescent substrate (Pierce) for the detection of HRP. Chemiluminescence was detected by exposure to Biomax film (Kodak Scientific).

2.4.6 Quantification of protein concentration – Bradford assay

Proteins were quantified using the Bradford assay (Bradford, 1976). Briefly, in a 96 well flat-bottom microtitre plate (Sarstedt), serial dilutions of each sample were made in a volume of 160 µl per well. The initial sample was added neat, and diluted 1:2 in each subsequent well. A standard curve was performed for each assay using bovine serum albumin (Fraction V) (Sigma) with a starting concentration of 1 mg/ml, diluted 1:2 in each subsequent well. Bradford reagent (40 µl) (Bio-Rad) was added to each well and mixed by pipetting. Absorbance was read at 570 nm wavelength on the Fluostar Optima (BMG Lab Technologies).

2.5 Histological and immunohistochemical techniques

2.5.1 Collection of tissues

Mice were humanely killed by CO_2 asphyxiation or cervical dislocation if older than 21 days, or by decapitation if younger. Individual tissues were collected, and either snap frozen in liquid nitrogen (for RNA or protein extraction) or fixed (for histological and immunohistochemical analyses) as described in Section 2.5.2. Human tissues were obtained from the Peter MacCallum Tissue Bank or commercially from SuperBioChips Laboratories (Korea).

2.5.2 Tissue processing

All tissues were fixed for 24 hours in 10 % neutral buffered formalin or 4 % (w/v) paraformaldehyde, and the fixative was removed by replacing with 70 % (v/v) ethanol. Fixed tissues were then dehydrated in increasing concentrations of ethanol, cleared with histosol, embedded in paraffin wax and sectioned (5 μm) using a Leica paraffin microtome. The tissue sections were floated in a 50°C water bath before being mounted onto ESCO Superfrost Plus slides (Biolab Scientific). Once on slides, the tissue sections were deparaffinised, by immersion in histosol twice for 5 minutes each. The slides were put through a rehydration series of 100 % (v/v) ethanol, 100 % (v/v) ethanol, 70 % (v/v) ethanol, and finally, dH_2O (5 minutes each), before being stained or used for immunohistochemistry.

2.5.3 Haemotoxylin and eosin staining

This stain was routinely used to examine tissue structures. The basic haematoxylin dye stains nuclear structures blue and the acidic eosin dye stains cytoplasmic and intercellular structures pink. Processed tissue sections were first immersed in Harris haematoxylin (BDH Biochemicals) (5 minutes), dipped briefly in acid-ethanol, immersed in Scott's blue (Fronine Laboratory Supplies) (1 minute), and then counterstained with eosin (BDH Biochemicals) (5 minutes). Between each step the slides were washed for five minutes under running tap water.

Following all staining procedures, the slides were put through a dehydration series of 70 % (v/v) ethanol, 100 % (v/v) ethanol, 100 % (v/v) ethanol and histosol (2 minutes each step), before they were cover-slipped using the permanent DePex mounting media (BDH

Biochemicals). The slides were viewed under a standard light microscope (Leica) at various magnifications.

2.5.4 Immunohistochemical staining

2.5.4.1 Antibody purification

Anti-Elf5 anti-peptide antibody was generated in our laboratory (J. Palmer, PhD Thesis 2003) by immunising rabbits against a peptide designed in the *ets* domain of ELF5 (described in Chapter 3). This peptide is common to human and mouse Elf5 proteins. Rabbit sera were affinity-purified on a Protein G sepharose column (Amersham Pharmacia Biotech). Neat serum was run twice through the column to bind IgG. The sepharose was washed with phosphate-buffered saline (PBS), eluted in 0.1 M glycine (pH 2.5) and neutralised with 0.1 volumes of 1 M Tris (pH 8.0).

2.5.4.2 Immunohistochemistry with the αElf5 antibody

Immunohistochemical detection was performed using the TSA-Indirect Kit (NEN Life Science Products) according to the manufacturer's instructions. Antigen retrieval involved treatment with 0.2 % (w/v) trypsin (Sigma) in 0.4 % (w/v) $CaCl_2$ for 10 min at 37°C. Endogenous peroxidases were quenched with 6 % (v/v) H_2O_2 (BDH Biochemicals) in PBS for 20 min. Affinity-purified anti-Elf5 rabbit sera was diluted 1:2500 in the blocking buffer supplied with the kit and incubated for 60 min at room temperature. Secondary antibody was a 1:200 dilution of biotinylated goat anti-rabbit IgG (DAKO) and incubation was for 40 min at room temperature. Visualisation utilised the DAB (3,3'-diaminobensidine) substrate kit (DAKO). Positive reactivity was demonstrated as a brown-coloured precipitate formed at the site of the target antigen. Sections were

counterstained with Harris haematoxylin (Section 2.5.3) and coverslips mounted with DPX (BDH Laboratory Supplies). For every tissue, a second serial section on the same slide was used as a negative control, and treated with affinity-purified pre-immune sera from the same rabbit.

2.6 Mammalian cell culture

2.6.1 Maintenance of mammalian cells in culture

The human breast carcinoma cell lines T47D, MCF7, SK-BR3, ZR-75-1, BT-474, MDA-MB-231, MDA-MB-435 and MDA-MB-453 and the human renal cell carcinoma line 786-O were grown in 1 X RPMI 1640 media (GibcoBRL) supplemented with 10 % (v/v) heat-inactivated Fetal Calf Serum (FCS) (CSL) and 1 % (w/v) Penicillin/Streptomycin (GibcoBRL). Cells were grown in a humidified incubator at 37°C and 5 % CO_2. Cells were subcultured at 1:2-10 ratio, with fluid renewal 2-3 times per week. Subculturing involved removal of media and washing the cells with PBS. Filter-sterilised 0.25 % (w/v) trypsin/EDTA (GibcoBRL) was added and incubated at 37°C for 1-5 minutes to loosen the cells' attachment to the flask. The trypsin was neutralised by addition of the media and a portion of the resultant mix transferred to each new flask, and supplemented with additional media. Long-term cell stocks were made by resuspending cell pellets in 95 % (v/v) FCS/5 % (v/v) dimethylsulfoxide (DMSO). The cells were transferred to a 1 ml cryotube (Greiner) and frozen at –80°C overnight in a polystyrene-insulated box. The cells were placed in liquid nitrogen for long-term storage.

2.6.2 Stable and transient transfections into mammalian cells

All stable and transient transfections were performed using the FuGene 6 reagent (Roche) according to the manufacturer's protocol. DNA for transfections was prepared using the Qiagen Endo-Free maxiprep kit. DNA (0.5 μg) was used to transfect 1-5x10^4 cells in 24-well plates for transient transfection experiments. For production of stable transfectants, 1 μg DNA was used to transfect 1x10^5 cells in 6-well plates. For all cell lines transfected, a 3:1 ratio of FuGene 6 (μl) to DNA (μg) was used.

2.6.3 Selection of stably transfected cell populations

Twenty four - 48 hours after cell transfection, selective antibiotic was added to the cells to kill untransfected cells. G418 (500 μg/ml) (Invitrogen), hygromycin B (400 μg/ml) (Roche) or puromycin sulphate (3 μg/ml) (Sigma) were found to be effective on all lines within 10-14 days or 5 days with puromycin selection. Colonies were then isolated and grown separately for further analysis including RNA (Section 2.3.2), DNA (Section 2.2.6) and protein (Section 2.4.1) preparation.

2.7 Statistical analysis

When data was analysed using one-way ANOVA, statistical analysis was performed using GraphPad Prism Version 2.0 (GraphPad Software Inc., San Diego, CA, USA). A value of $p<0.05$ was considered statistically significant.

Chapter 3. Expression of the ets transcription factor, Elf5, is restricted to glandular epithelium

3.1 Introduction

3.1.1 *ELF5* is an *ets* transcription factor

The *Elf5/Ese-2* gene is a member of the *ets* family of winged-turn-helix transcription factors that recognise the consensus sequence GGA(A/T) found in the promoters and enhancers of many genes. Most *ets* transcription factors are expressed either ubiquitously or in haemopoietic cells and organs. In these cell types, *ets* genes such as the archetypal family member, *ets-1*, are crucial for maintaining a normal differentiated cell phenotype by integrating extracellular signals at the transcriptional level, resulting in the activation of a large set of target genes (Wasylyk et al., 1993). However, members of the epithelial-specific ets (ESE) subfamily, *Elf5/Ese-2* (hereafter *Elf5*), *Elf3/Ese-1/Ert/Esx/Jen*, *Ehf/Ese-3* and *Pdef/Pse*, are expressed only in organ systems rich in epithelium (Chang, 1997; Kas et al., 2000; Kleinbaum et al., 1999; Nozawa et al., 2000; Tymms et al., 1997; Wasylyk et al., 1993; Yamada et al., 2000; Zhou et al., 1998).

3.1.2 Expression analysis of 'epithelial-specific' *ets* factors

Initial expression analysis of *Elf5*, *Elf3*, *Ehf* and *Pse* was undertaken using RNA transcript analysis on whole human and/or mouse organ lysates. Protein expression analysis using

immunohistochemistry has been published only regarding PSE, establishing the restriction of this factor to the prostatic glandular epithelium (Nozawa et al., 2000). Differences in the expression patterns of the *ESE* genes have been identified at the transcript level that may contribute to the different functions of the genes. *Elf3*, which transactivates epithelial-specific target genes such as *Keratin-4*, *SPPRAA2A*, *Endo-A* and *Her2*, is expressed in a wide variety of simple and stratified epithelial tissues with highest expression in the gastrointestinal epithelium and involuting mammary gland (Brembeck et al., 2000; Chang, 1997; Neve et al., 1998; Oettgen et al., 1997b; Tymms et al., 1997). *PSE* is translated only in prostatic epithelium and regulates *prostate-specific antigen (PSA)* gene expression (Chen et al., 2002; Oettgen et al., 2000). *Elf5* mRNA is present in a more restricted set of tissues encompassing the salivary and mammary glands, lung, kidney, bladder, stomach and prostate (Oettgen et al., 1999b; Zhou et al., 1998). *Ehf* is most highly expressed in similar tissues to *Elf5* but is also expressed at lower levels in additional organs (Kleinbaum et al., 1999).

Compared to *Elf3*, *Ehf* has different binding affinities for a variety of epithelial-specific promoters and transactivates the *PSA* promoter where *Elf3* transrepresses it (Kas et al., 2000). Likewise, while both *Elf3* and *Elf5* recognise an *ets* site in the mammary specific *whey acidic protein (WAP)* promoter, they transactivate to vastly different degrees (Thomas et al., 2000). This indicates that an *ets* factor may promote or repress transcription on a target promoter depending on its interaction with other proteins on the DNA. Specificity for a cognate *ets* motif is also determined by sequences flanking the core recognition sequence GGAA/T. The combined effect of the changes in the flanking sequence appears

to contribute towards *ets* factor binding specificity (Oettgen et al., 1999b; Wang et al., 1992; Wasylyk et al., 1992). Therefore, despite the highly conserved DNA-binding domains and overlapping spatial and temporal expression patterns of these genes, their functions *in vivo* are apparently distinct.

To address the question of whether *Elf5*, a member of the 'ESE' subfamily, is expressed in an epithelial-restricted manner, we have generated a specific anti-peptide antibody to perform *Elf5*-specific immunohistochemical localisation in mouse tissues. Our experiments show that in adult mice, Elf5 protein is restricted to certain subsets of epithelial cells that have a secretory or glandular function.

3.2 Methods

3.2.1 Production of an anti-Elf5 peptide antibody

A 12 amino acid peptide in the *Elf5* DNA-binding domain was examined for hydrophilicity, flexibility and antigenicity using the MacVector (Version 6.5; Accelrys) software program. The sequence, CGILEWEDREQG, identical in mouse and human homologues, was synthesised and conjugated to the diphtheria toxoid carrier protein via a maleimidocaproyl-N-hydroxysuccinimide (MCS) linker utilising a native cysteine residue at the N-terminus of the peptide (Mimotopes Pty Ltd). Peptides were RP-HPLC purified to a purity of >70%. Two New Zealand white rabbits were immunised initially with 250 nmol conjugated peptide in 500 µl of Freund's complete adjuvant (Sigma), followed by three booster immunisations of 250 nmol conjugated peptide in 500µl Freund's incomplete

adjuvant (Sigma). Four weeks after the final immunisation the rabbits were sacrificed by exsanguinations by Central Animal House staff (Monash University). Peptide design was performed by Jodie Palmer.

3.2.2 ELISA

The pre-immune and immune sera were analysed by Jodie Palmer for the presence of anti-*Elf5* antibodies using standard ELISA techniques as described by the peptide manufacturers (Mimetope Chiron Pty Ltd, 1995). Titre was determined by colourimetric change at 450nm with a reference of 495nm in the Biorad 3550 microplate reader.

3.2.3 Western analysis

Recombinant proteins were produced in *E. coli* using the PQE30 vector (Qiagen) containing a 6xHistidine tag at the C-terminus of the protein. Samples containing 200 ng recombinant protein, together with molecular weight standards, were subjected to SDS-PAGE on 12 % (w/v) acrylamide gels under reducing conditions, and the proteins were transferred to Immobilon-P membranes (Millipore Corporation) according to the manufacturer's instructions. Purified rabbit IgG was used at 1/1000 dilution and secondary goat anti-rabbit IgG conjugated to HRP (DAKO Corporation) at 1/1000 dilution in 5 % (w/v) skim milk powder/TBST. Chemiluminescence was detected using the SuperSignal reagent (Pierce Endogen). Blots were exposed to Kodak X-OMAT AR film for up to 2 min and developed.

3.2.4 Immunohistochemistry

Rabbit sera were affinity-purified on a Protein G sepharose column (Amersham Pharmacia Biotech). Neat serum was run twice through the column to bind IgG. The sepharose was washed with PBS, eluted in 0.1 M glycine (pH 2.5) and neutralised with 0.1 volumes of 1 M Tris (pH8.0). Murine organs were dissected from C57Bl6xSV129J mixed background mice, rinsed in cold PBS and fixed overnight in 10 % neutral-buffered formalin. Fixative was removed by replacing with 70 % (v/v) ethanol. Organs were processed and embedded in paraffin wax and cut in 5 µm sections onto Superfrost Plus slides (Menzel-Glaser). Immunohistochemical detection was performed using the TSA-Indirect Kit (NEN Life Science Products) according to the manufacturer's instructions. Antigen retrieval involved treatment with 0.2 % (w/v) trypsin in 0.4 % (w/v) $CaCl_2$ for 10 min at 37°C. Endogenous peroxidases were quenched with 6 % (v/v) H_2O_2 in PBS for 20 min. Affinity-purified anti-Elf5 rabbit sera was diluted 1:2500 in the blocking buffer supplied with the kit and incubated for 60 min at room temperature. Biotinylated goat anti-rabbit IgG (DAKO) secondary antibody was used at 1:200 dilution and incubated at room temperature for 40 minutes. Visualisation utilised the DAB substrate kit (DAKO). Sections were counterstained with Harris haematoxylin and coverslips mounted with DPX (BDH Laboratory Supplies). For every tissue, a second serial section on the same slide was used as a negative control, and treated with affinity-purified pre-immune sera from the same rabbit.

3.2.5 Production of recombinant Ets protein

PQE-30 (Qiagen) constructs encoding human ELF5 p20 and p30 (Jiong Zhou), murine Elf5 (Jiong Zhou), human ELF3 (Annie Ng), human ETS-1 and ETS-2 (Ross Thomas) were previously constructed in our laboratory. Murine full-length *Ese-3* cDNA was amplified from kidney cDNA with oligonucleotides 4 and 5 (Appendix I) and inserted into the *Bam*HI and *Sac*I sites of the pET-21b+ vector (Clontech).

3.3 Results

3.3.1 Production of a rabbit anti-peptide antibody against Elf5

The peptide against which the antibody was directed is unique to ELF5 (Figure 3.1). The most closely conserved sequence is found in ESE-3, but the antibody does not cross-react with recombinant ESE-3 (Figure 3.2). Anti-Elf5 sera from one of the rabbits specifically recognised the Elf5 recombinant proteins in a denatured form on a Western blot. It did not cross-react with other ets family members including Ets-1 and Ets-2 (Figure 3.2). Pre-immune sera from the same rabbit did not recognise these proteins (data not shown). Antibody binding was reduced when the sera had been pre-incubated with *Elf5* peptide, indicating the specificity of the interaction.

3.3.2 Immunolocalisation of Elf5 protein to epithelial cells in murine kidney, mammary gland, parotid gland and stomach

Cell types staining positive for Elf5 protein were identified by morphology. Kidney epithelium, but not the non-epithelial glomeruli, showed positive staining representing presence of the Elf5 protein (Figure 3.3A). Expression was most easily detectable in the kidney cortex both in the nucleus and cytoplasm of cells. In the medulla, staining was less intense and primarily confined to cell nuclei. Both ductal and alveolar epithelium in the mammary gland stained positive (Figure 3.3B). In the submandibular salivary gland, Elf5 staining was observed in the secretory alveoli that produce mucus and serous fluid (Figure 3.3C). In the stomach, a gradient of Elf5 expressed was observed along the crypto-villus axis, with strong, nuclear staining observed in the base of the crypts (Figure 3.3D), decreasing in intensity and cell number along the villus. No cells were stained at the tip of the villi. Tissues found to be expressing *Elf5* mRNA, including keratinocytes (Oettgen et al., 1999b), bladder, lung, trachea and prostate (Zhou et al., 1998) were also analysed by immunohistochemistry but the protein was not detected.

```
A     162   SS----------------------------------------------------------HLW
B     160   SS----------------------------------------------------------HLW
C     228   PSDGFRDCKKGCP-------KHGKRKRGRPRKLSKEC---WDCLEGKKSKHAPRGTHLW
D     183   ESPDMKKEQDPPA----------ECH-------------------TKKHNP--RGTHLW
E     281   PSYDSTDSEDYPAALPNHKPKGTFKDYVRDRADLNKD-KPVIPAAALAGYTGSGPIQLW
F     309   PSGESGEDDCSQS-LCLNKPTMSFKDYIQERSKPVEQGKPVIPAAVLAGFTGSGPIQLW

A     167   EFVRDLLLSPEEN          IFRVVKSEALAKMWGQRKKNDRMTYEKLSRALRY
B     165   EFVRDLLLSPEEN          IFRVVKSEALAKMWGQRKKNDRMTYEKLSRALRY
C     276   EFIRDILIHPELNE LMK  N HE VFKFLRSEAVAQLWGQKKKNSNMTYEKLSRAMRY
D     211   EFIRDILLNPDKNP LIK    SE VFRFLKSEAVAQLWGKKKNNSSMTYEKLSRAMRY
E     339   QFLLE-LLTDKSCQSFIS TG-DGWEFKLSKPDEVARRWGKRKNKPKMNYEKLSRGLRY
F     367   QFLLE-LLSDKSCQSFIS TG-DGWEFKLADPDEVARRWGKRKNKPKMNYEKLSRGLRY

A     226   YYKTGILERVD--RRLVYKF
B     224   YYKTGILERVD--RRLVYKF
C     336   YYKREILERVD-GRRLVYKF
D     270   YYKREILERVD-GRRLVYKF
E     396   YYDKNIIHKTA-GKRYVYRF
F     424   YYDKNIIHKTS-GKRYVYRF
```

Figure 3.1. Alignment of the ETS domain sequences of ESE proteins, ETS-1 and ETS-2. Genbank accession codes are bracketed. A) human ELF5 (AAC79755); B) murine Elf5 (AAC79754); C) ELF3 (AAM70481); D) Ese-3 (NP_031940); E) ETS-1 (NP_005229) and F) ETS-2 (NP_005230). The numbers along the left side of the sequence indicate the amino acid residue position, taken from the cited Genbank reference. The peptide used to immunise mice is highlighted in pink. Residues conserved among the related factors are highlighted in blue.

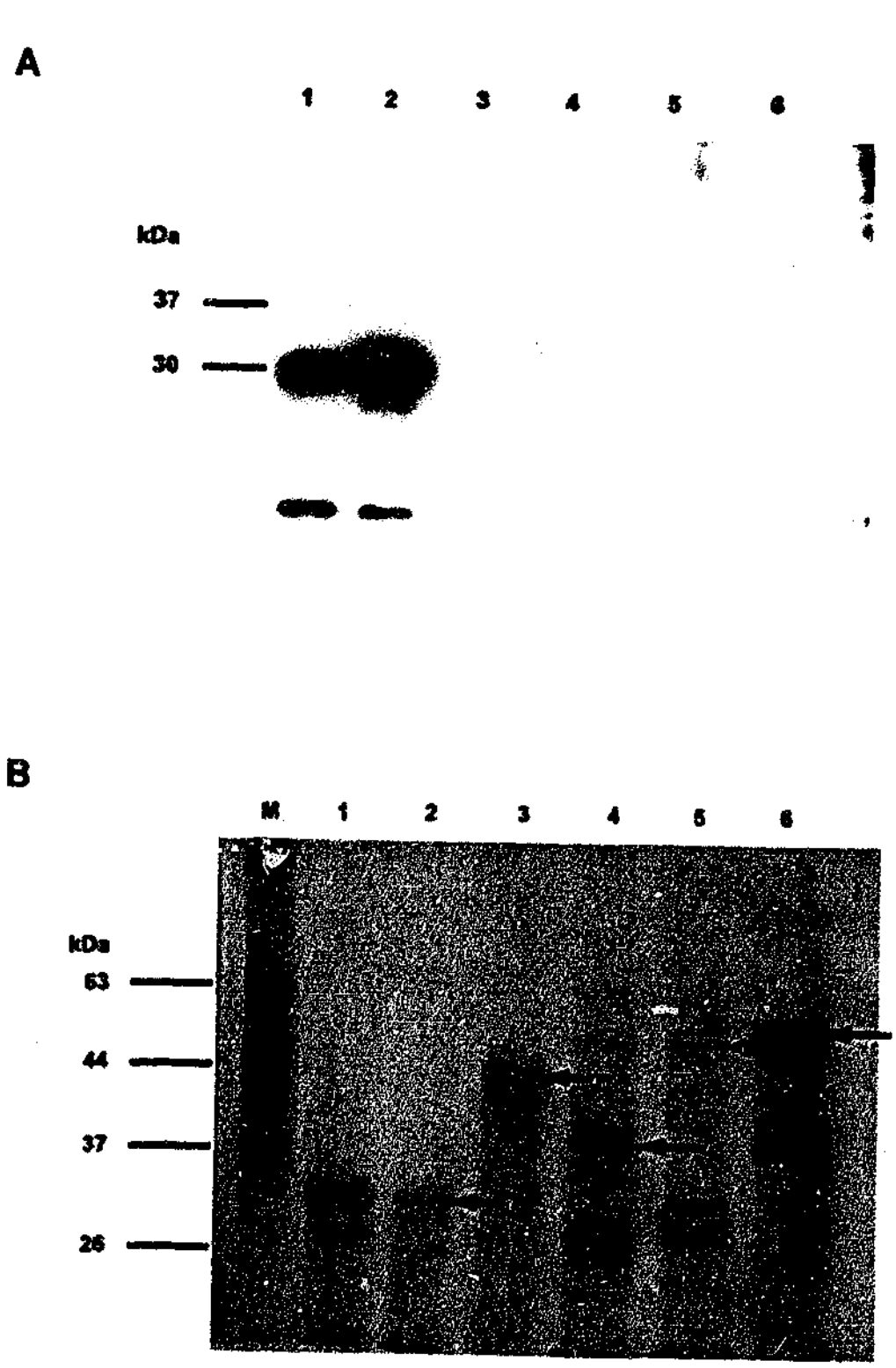


Figure 3.2. The ELF5-peptide antiserum is specific for Elf5. Recombinant proteins shown: 1) human ELF5; 2) murine Elf5; 3) Elf3; 4) Ese-3; 5) ETS-1 and 6) ETS-2 were analysed by Western blot with Elf5 peptide antibodies (A) and their presence on the gel was verified by silver-staining (B).

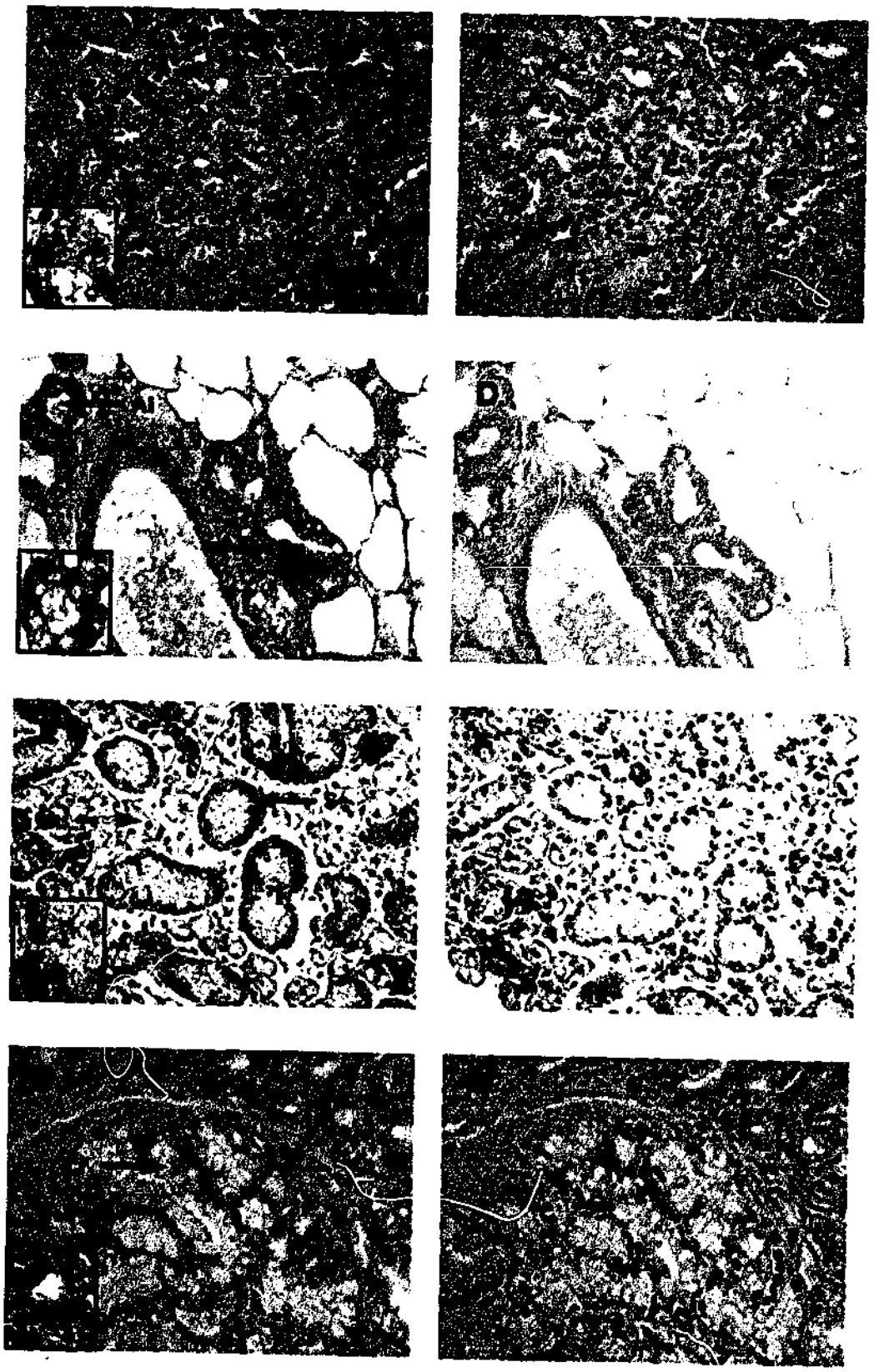


Figure 3.3. Elf5 protein is localised to secretory epithelia. Immunohistochemistry with anti-Elf5 peptide antibody (A, C, E, G) or preimmune sera (B, D, F, H) was performed on sections of murine kidney (A, B), day 1 lactating mammary gland (C, D), submandibular gland (E, F) and stomach (G, H). Brown staining indicates positive staining. Features are marked as glomerulus (Gl), alveolus (Al), duct (Du), mucinous epithelium (Mu), serous epithelium (Se) and base of crypt (Cr).

3.4 Discussion

3.4.1 *Elf5* is epithelial-specific

This study has shown for the first time, using highly sensitive immunohistochemical techniques, that expression of the 'epithelial-specific' *ets* gene *Elf5* is indeed localised to subsets of secretory epithelial cells in the kidney, mammary gland, parotid gland and stomach. *Elf5* was expressed in most epithelial cells in the kidney and mammary gland, but only in one of the two epithelial lineages in the submandibular gland, and in the proliferating epithelial cells at the base of the crypt in the stomach. This contrasts with its elevated mRNA expression in differentiated human primary foreskin keratinocytes (Oettgen et al., 1999b) and suggests that Elf5 performs different functions in different epithelial lineages and is not, *per se*, required for a terminally differentiated epithelial phenotype, at least in the cells of the stomach lining.

3.4.2 *Elf5* is not translated in all the organs in which it is transcribed

Interestingly, Elf5 protein was not detectable by immmunohistochemistry in other organs in which its mRNA is expressed. Messenger RNA was detected in human bladder, lung and prostate, and in murine bladder and lung. Murine prostate was not examined, so it is possible the transcript is not expressed here, though in all other organs the human and murine expression patterns match (Oettgen et al., 1999b; Zhou et al., 1998). The phenomenon of RNA and protein product expression patterns differing for a gene has been observed regarding other *ets* transcription factors, particularly *PSE*, where mRNA can be expressed in all prostatic glandular epithelium but translation takes place most efficiently in

normal cells, but not at all in malignant cells. Translational control of this transcript was shown to be due to regions in the 5'UTR and 3'UTR (Nozawa et al., 2000). As two transcripts of *Elf5*, with differing 3'UTR sequences, have been identified (Zhou et al., 1998), this form of translational control is a distinct possibility for the Elf5 protein.

3.4.3 Conclusions

We have produced an antiserum that specifically detects Elf5 protein and shown localisation of the protein to glandular epithelium. The antibody can be used as a reagent to further analyse the expression of Elf5 protein in human and mouse and to assist in elucidating the function of Elf5 in development and disease.

The work in this chapter is under review for publication in *Histochemistry and Cell Biology*.

Chapter 4. Transcriptional regulation of ELF5: STAT1 and NFκB regulate *ELF5* in breast cancer cell lines

4.1 Introduction

4.1.1 Transcriptional regulation of *Elf5* in the murine mammary gland

Murine *Elf5* transcript levels are temporally regulated during pregnancy-associated mammary gland development (Zhou et al., 1998). As the epithelial cells of the terminal end buds (TEBs) proliferate and differentiate, *Elf5* mRNA levels increase, peaking at d12 of pregnancy. They remain high throughout the remainder of the pregnancy and are stable during lactation (Appendix VII). As the gland involutes, however, *Elf5* transcript decreases while levels of the closely related transcription factor, *Elf3*, concomitantly increase (Neve et al., 1998). Therefore, *Elf5* mRNA levels are transcriptionally regulated during mammary gland development, and are highest during the phases of TEB epithelial growth and the differentiation of these cells into secretory alveoli. This suggests a role for *Elf5* in these processes. Given the almost complete similarity of human and murine *ELF5* coding sequences and the close alignment of their expression patterns (Oettgen et al., 1999b; Zhou et al., 1998), it is likely that *ELF5* is also transcriptionally regulated in pregnancy-associated breast development in the human. However, while *ELF5* mRNA has been detected by Northern hybridisation in the

human breast (Ma et al., 2003a; Oettgen et al., 1999b), no data is available on whether it is regulated like the mouse homologue in development.

4.1.2 Transcriptional dysregulation of *ELF5* in human breast cancer

In the human, *ELF5* appears to be dysregulated in breast cancer and breast cancer cell lines at the transcriptional level, as the mRNA is either downregulated or undetectable (Ma et al., 2003a; Zhou et al., 1998). Characterising a promoter region for this gene will assist in understanding mechanisms by which this may occur. Firstly, identification of factors that regulate the promoter will provide information on which pathways *ELF5* might be involved in. That is, if *ELF5* is regulated by factors that regulate other growth and differentiation associated genes, it places it in a signalling response pathway with the co-regulated genes. Secondly, for future studies, if the promoter of *ELF5* is epigenetically altered in cancer or even mutated, this may provide a rationale for the decrease of *ELF5* expression in cancer and hence provide further evidence for its role as a tumour suppressor gene.

Ma and colleagues (2003) have shown the down-regulation of *ELF5* mRNA in primary breast cancers - in high proportions of early and mid-stage cancers, and in all of the high-grade cancers in the study sample. Several possibilities exist that could explain this phenomenon – LOH at the locus, downregulation of expression by changes in chromatin structure, histone acetylation and promoter methylation, or alterations in expression of the upstream genes responsible for the maintenance of *ELF5* transcription. Investigation into these possibilities requires the characterisation of the promoter region for this gene.

4.1.3 Analysing the *ELF5* promoter

Here we report the first isolation and analysis of the *ELF5* promoter region, and take a candidate approach to elucidating potential upstream regulators of the gene. Using the luciferase reporter system with deletion mutants of the putative 5' regulatory regions of *ELF5* (R. Chehab, Honours Thesis, 2002), we have shown the location of active promoter regions, and that the deletion of potential regulatory motifs can increase and decrease promoter activity. This implies the presence of repressor and enhancer elements in certain locations. The minimal promoter element has been localised to a small genomic region associated with the first, non-coding exon (R. Chehab, Honours Thesis, 2002). The first intron, which was presumed to be a second promoter specific for a second transcriptional isoform of *ELF5* (Oettgen et al., 1999b), is not active in driving transcription. We have tested the functionality of several predicted regulatory motifs in the promoter, particularly the cognate motifs of transcription factors with an established role in cancer. These include the steroid hormone signalling pathways stimulated by estrogen and progesterone (Chapter 5); NFκB, which is over-expressed or inappropriately activated in prostate and breast cancers (Abdel-Mageed and Agrawal, 1998; Kikuchi et al., 2003; Li and Li, 2001; Varela et al., 2001); *ELF5* as a potential autoregulating protein; *ELF3* as an *ets* factor that is over-expressed in breast cancer (Chang, 1997); the general transcription factor, AP-1; and the signal transducer and activation of transcription (STAT) proteins that modulate the Janus-associated kinase (JAK) pathways. These are associated with mammary gland development in pregnancy and also with constitutive activation in breast cancer, among other cancer types (Berclaz et al., 2001; Li and Shaw, 2002; Llovera et al., 2000; Schaber et al., 1998; Watson, 2001; Widschwendter et al., 2002; Zhang et al., 2003).

Studying the transcriptional regulation of *ELF5* is important to better understand the remodelling of the mammary gland tissue architecture. It is also crucial to elucidating the mechanism by which *ELF5* transcript levels are altered in breast epithelial cancer.

4.2 Methods

4.2.1 Characterisation of the genomic structure of *ELF5*

RPC11 *ELF5*-containing bacterial artificial chromosomes (BACs) were obtained from ResGen (Invitrogen). These were directly sequenced with exon-specific primers to obtain sequence surrounding the exon splice junctions. Additionally, PCR between exons was used to amplify intronic regions. When this analysis was partially complete, the incomplete genomic sequence of human ELF5 was placed on the public human genomic contig database (http://www.ncbi.nlm.nih.gov/entrez) (AL137224). This has since been completed, and used as a template for further inquiries. Intron/exon boundary sequences were delineated by comparing the genomic locus sequence to the known cDNA sequence of *ELF5* (NM_001422).

4.2.2 Generation of h*ELF5* promoter fragments

Based on the AL137224 sequence, six primers were designed in the 6 kb region 5′ to *ELF5* exon 1. PCR amplimers were generated from these positions to terminate 3′ of exon 1. Several shorter amplimers were also generated, encompassing different parts of the region 5′ to exon 1, exon 1 itself and intron 1 (Figure 4.1). Normal human genomic DNA (100 ng) extracted from blood was used as a PCR template. The relative positions of the PCR primers that initiate and terminate the fragments are enumerated on Figure 4.1 and the sequences given in Appendix I (oligonucleotides 6-15).

All promoter fragments were A-tailed and cloned into the pGEM-T vector (Promega) and subsequently subcloned into the EGFP reporter vector (Clontech). The intron 1 inserts were excised from pGEM-T with *Nco*I and blunted, then re-digested with *Sac*II. Inserts were sub-cloned into the *Sac*II/*Sma*I sites of the pEGFP vector. The promoter inserts were excised from pGEM-T with *Sal*I, blunt-ended and re-digested with *Sac*II. Inserts were cloned into the *Sac*II/*Sma*I sites of pEGFP. All clones were sequenced using the EGFP-N (5′-CGTCGCCGTCCAGCTCGACCAG-3′) and EGFP-C (5′-CATGGTCCTGCTGGAGTTCGTG-3′) sequencing primers.

4.2.3 Serial deletion of the 1 kb promoter + exon 1 DNA

Following the discovery of a functional promoter element in the 1 kb promoter + exon 1 fragment (Fragment F-G, Figure 4.1), a series of smaller deletions were made from the 5′ end of this DNA to attempt to isolate a minimal promoter region. The deletions were made using convenient 5′ restriction endonuclease sites (see Figure 4.4A) and the *Bam*HI site at the 3′ end, and the resultant fragments cloned into the *Xho*I/*Bgl*II sites of the pGL3 basic luciferase reporter vector (Promega). These constructs were expressed in T47D cells for quantification of reporter gene activity (R. Chehab, Honours Thesis, 2002).

4.2.4 Putative transcriptional regulators of the h*ELF5* promoter

ELF5, *ELF3*, *AP-1* subunits *c-jun* and *c-fos*, *STAT1*, *STAT3*, *STAT5a* and *STAT5b* cDNAs in the pEF1-BOS plasmid (Mizushima and Nagata, 1990), and NFκB subunits p50 and p65 in the pCI vector (Promega) were expressed transiently in MCF7 and T47D cells to determine whether they affected the level of transcription from the *ELF5*

promoter. The *STAT* constructs were kindly supplied by Warren Alexander (Walter and Eliza Hall Institute, Melbourne, Australia). *NFκB* subunits were a gift of Peter Oettgen (Beth Israel Deaconess Medical Centre, Harvard Medical School, Boston). All other expression constructs were made in this laboratory by Ross Thomas.

4.2.5 Transfection into T47D and MCF7 cells

T47D and MCF7 breast cancer cells were cultured in RPMI 1640 (Invitrogen) with 10 % (v/v) FCS in a 37°C humidified incubator with 5% CO_2 in air. MCF7-derived cell lines that stably express *ELF5* are described in Chapter 6. For transfection of the promoter-EGFP reporter constructs, $5x10^5$ cells were plated on a 0.2 % (w/v) gelatin-coated coverslip in 6-well plates. For each plasmid to be transfected, six wells were plated: three for direct visualisation of EGFP in cells and three for homogenisation to assay for β-galactosidase enzyme activity, used as a control for plasmid transfection efficiency. The pSV-βgal plasmid (Promega) was used as a transfection efficiency control. Per well, each transfection consisted of 1 μg of DNA, where 5/6 was made up by the relevant h*ELF5-EGFP* reporter plasmids and 1/6 by pSV-βgal DNA. The pEGFP-NI (Clontech) plasmid was used as positive control for EGFP fluorescence, where EGFP is driven by the CMV promoter, and promoterless EGFP was used as a negative control. For transfection of the pGL3 luciferase reporter constructs, $5x10^4$ cells were plated in 24 well plates (Falcon). DNA (0.5 μg) was transfected per well, consisting of 1/6 pSV-βgal DNA and then 5/6 of the largest luciferase reporter construct. To transfect the smaller constructs, an equimolar amount of construct was added and the remainder of the 0.5 μg made up with promoterless pGL3 DNA. DNA was prepared using the Qiagen Endo-Free maxiprep kit (Qiagen). Transfections were

carried out using FuGene 6 transfection reagent (Roche Molecular Biochemicals) and incubated for 48 hours. The transfections using the luciferase reporter constructs were performed by Renēe Chehab. Where relevant, cells were treated with ovine prolactin to induce a differentiated phenotype. Four hours post-transfection, cells were serum starved in unsupplemented RPMI 1640. After 24 hours, media was replaced with RPMI 1640 supplemented with 10 % (w/v) FCS and 1 μg/ml prolactin.

EGFP transfected wells were tested for EGFP marker gene expression by fluorescence microscopy using a GFP (488nm) filter. Nuclei were counterstained with 1 ng/ml 4'-6-diamidino-2-phenylindole (DAPI) in PBS (Sigma) for 10 min and visualised under UV light microscopy. Luciferase assays were harvested using the Constant Light Signal Luciferase kit (Roche). Luciferase activity was measured on the Lumicount (Hewlett-Packard) then 50 μl of lysate was incubated with an equal volume of 2 X β-galactosidase sample buffer (4.5 mM 2-nitrophenyl-β-D-galactopyranoside, 0.08 % (v/v) β-mercaptoethanol, 2 mM $MgCl_2$, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4) at 37°C until colour had developed. Absorption was measured at 415 nm on the BioRad 3550 plate reader. Results were normalised by dividing luminescence units by the β-galactosidase assay readout. All transfection experiments were performed at least three times in triplicate, so that n>9.

4.2.6 Computer analysis of functional promoter regions

The smallest active promoter region discovered in the EGFP assay (1 kb promoter + exon 1) was analysed for putative transcription factor binding sites. Searches were performed using the Baylor College of Medicine (BCM) Search Launcher program

TESS (http://searchlauncher.bcm.tmc.edu), and MatInspector (http://genomatix.gsf.de/mat_fam). Alignment of human and mouse genomic loci was performed using AVID, with a window size of 100 bp and a conservation level of 70 %. Results were viewed in VISTA (http://www-gsd.lbl.gov/vista/). Genomic comparisons were performed by Reneē Chehab.

4.3 Results

4.3.1 Mapping the h*ELF5* genomic structure

The genomic structure of the *ELF5* locus on chromosome 11p13 is shown in Figure 4.2A. The resultant full-length (p30) protein, produced from the transcript containing exons 1 and 2 (but not exon 1A), is indicated below (Figure 4.2B). Here, all nucleotide positions are named relative to the exon 2 translational start codon (+1) from which both p30 and p20 isoforms are made. Alternative splicing isoforms of *ELF5* are further discussed in Chapter 5. The 3′ splice acceptor sequences are shown in Table 4.1 and conform to the MAG/*gt*ragt consensus, where the italicised nucleotides are invariant. Lower-case letters denote intronic sequence and upper-case exonic sequence. The 5′ splice donor sequences conform to the yyyyyyyyyyyny*ag*/G consensus, except in exon 3 where the *ag* has been replaced by a *tg* dinucleotide. However, the splice junction is still functional, as the exon forms part of the *ELF5* transcript.

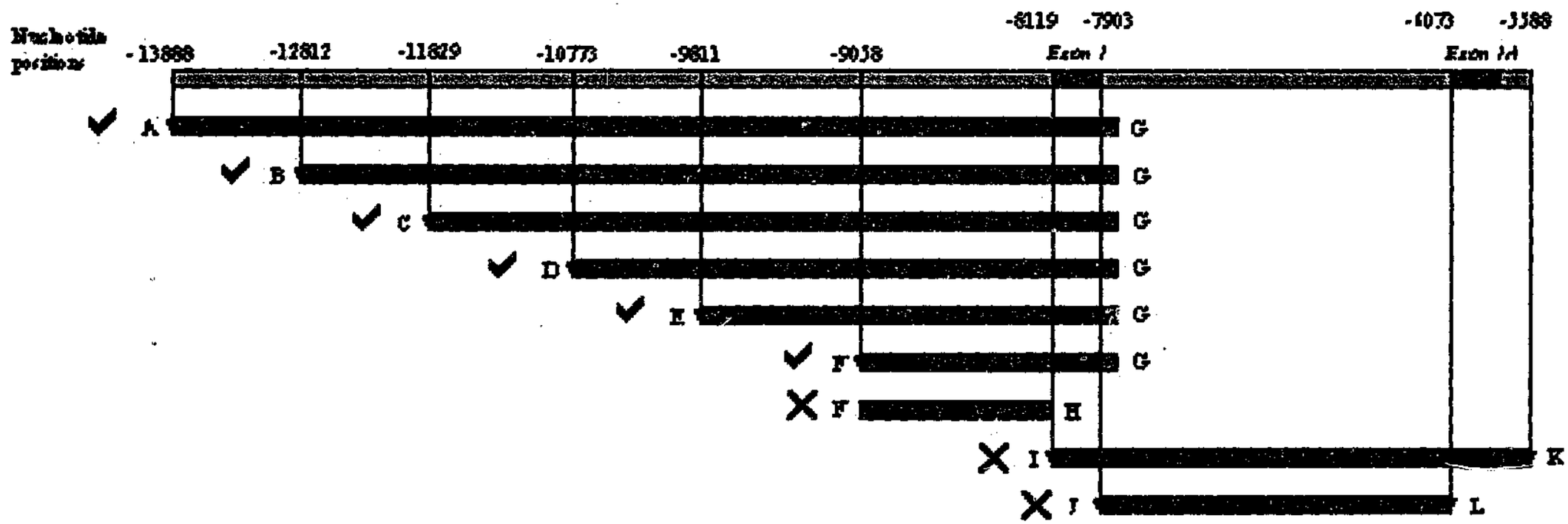
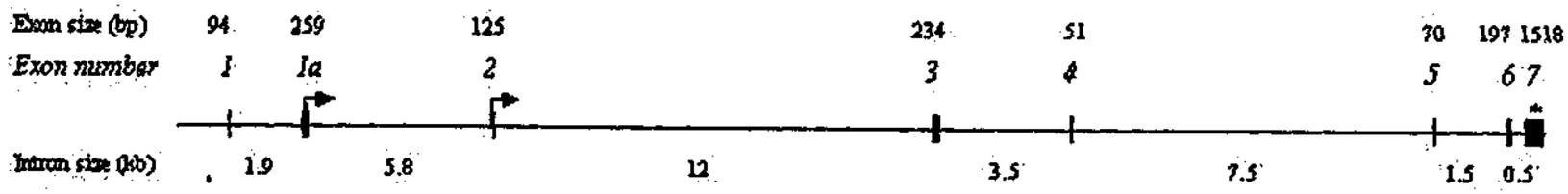


Figure 4.1. Putative promoter fragments PCR amplified and cloned into the pEGFP reporter vector. Nucleotide positions of the start of each fragment are indicated relative to the +1 translational start site of the major *ELF5* p30 transcript in exon 2. Blue colouring indicates non-transcribed DNA and green colouring indicates exons. A tick next to the fragment indicates its functionality in driving EGFP expression in T47D cells while a cross indicates no EGFP visualisation in cells transfected with that reporter construct.

4.3.2 Isolation of a functional promoter region for *ELF5*

Large deletions of the putative 5′ regulatory region, including the non-coding exon 1, were generated by PCR, starting approximately 6 kb 5′ to exon 1 and progressively deleting about 1 kb with each successive construct. These fragments were used to drive the EGFP reporter gene in T47D cells, which express *ELF5* endogenously and hence possess all the regulatory factors necessary for *ELF5* transcription (Zhou et al., 1998). The first intron, both with and without exon 1, was also tested in this assay. These fragments are shown in Figure 4.1A. Transiently transfected cells were visualised for EGFP fluorescence and DAPI fluorescence to detect nuclei, showing that cells were present in the field of vision. Promoterless EGFP was used as a negative control, and showed no fluorescence associated with cells. pEGFP-NI (utilising the CMV promoter) was used as a positive control for EGFP expression. All constructs utilising the *ELF5* 5′ region including exon 1 showed promoter activity as demonstrated by EGFP fluorescence in T47D cells. An example is shown in Figure 4.3. Deletion of exon 1 ablated fluorescence. Constructs using the intron 1 region, regardless of whether they contained exon 1 or 1A sequences, appeared to be inactive as promoters as no EGFP was visible. Transfection efficiency was controlled for by measuring β-galactosidase activity. All samples had comparable transfection efficiencies (data not shown).

A



B

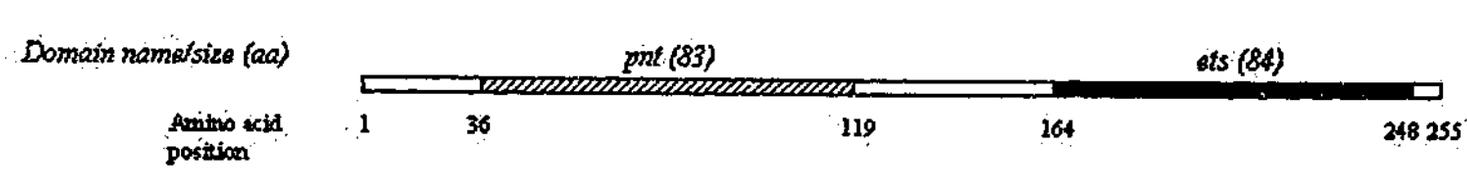


Figure 4.2. Genomic structure of the h*ELF5* locus and domains of the resultant protein product. Exons 1 and 1A are alternative first exons. Not to scale. The major, p30, *ELF5* transcript is produced from the translational start codon in exon 2 (A). *ELF5* alternatively spliced transcripts are discussed further in Chapter 5. Translation of the major transcript results in a 30 kDa protein that possesses both *pointed* and *ets* domains (B).

4.3.3 Analysis of the 1 kb promoter + exon 1 region

Further analysis was undertaken on the 1 kb promoter + exon 1 regions (Fragment F-G, Figure 4.1) as it was the smallest DNA fragment functional as a promoter in T47D cells. Smaller 5' end deletions of this fragment, consisting of 100-300 base pairs, were made using convenient restriction endonuclease sites. The resultant DNAs (Figure 4.4A) were cloned into the luciferase reporter vector and expressed in T47D cells to quantify their activity as promoters (Figure 4.4B). This work was performed by Renēe Chehab. The F-G promoter fragment was scanned for predicted transcription factor binding sites by computer programmes TESS and MatInspector. Output from these searches is located in Appendix V. Predicted binding sites that, when deleted, correlated with changes in promoter activity were noted to be putative transcriptional repressor and enhancer elements. Figure 4.5 shows potentially interesting binding sites and their relative distribution on the F-G region.

ELF5 Exon Boundary Sequences			
	5′ Splice Donor	3′ Splice Acceptor	Position relative to +1
Consensus	yyyyyyyyyynyag/G	MAG/gtragt	
Exon 1		CAC/gtaagc	-8021/-7928
Exon 1A	ggcaaactggccaag/G	CAG/gtacct	-6049/-5795
Exon 2	ctcctgctgttgcag/G	CAG/gtgcgt	-4/+120
Exon 3	ctttctcctttggtg/C	AAG/gtcagt	+12031/+12267
Exon 4	ctttttctttcccag/G	ACT/gtaagt	+15687/+15737
Exon 5	attattctatttcag/A	CAA/gtaagt	+23247/+23315
Exon 6	tccccttttcaaaag/G	GAG/gtaagt	+24779/+24974
Exon 7	tgcttgcttttcag/A		+25432/+26952

Table 4.1. *ELF5* exon boundary sequences conform to the human splicing consensus sequences. The exon 3 5′ splice donor sequence has a change in a normally invariant nucleotide, but the junction is still functional. Nucleotides marked in blue are normally invariant from the consensus sequence. Y = C/T; M = A/C; R = A/G; N = A/T/C/G. Position of the exons relative to the +1 translational start site of exon 2 are shown as first exonic nucleotide/last exonic nucleotide.

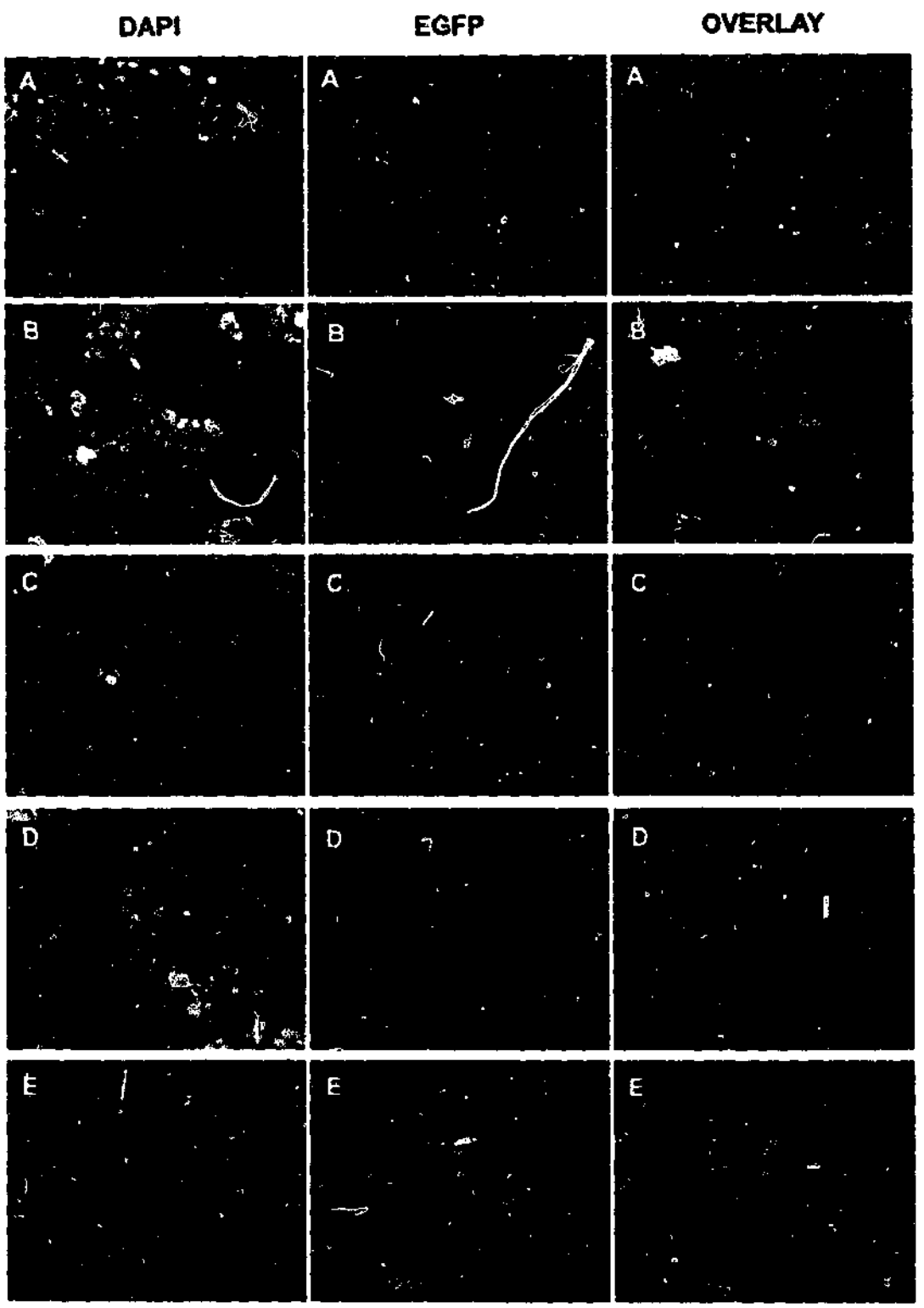


Figure 4.3. h*ELF5* promoter-EGFP constructs were transiently transfected into T47D cells and visualised for EGFP fluorescence and DAPI incorporation to display nuclei. Intron 1 + exon construct shows no fluorescence (A) while the 4 kb promoter + exon 1 (B) and 1 kb promoter + exon 1 (C) construct transfectants show fluorescent cells. Promoterless EGFP (D) is a negative control for fluorescence while EGFP under the strong, ubiquitous CMV promoter (E) acts as a positive control.

Activity of the full-length F-G (*Xho*I) fragment showed a 47-fold increase over the promoterless pGL3 basic vector (R. Chehab, Honours Thesis, 2002). Significant reductions in luciferase activity occur in the *Bst*EII and *Dra*I fragments compared to the F-G fragment, coincident with the loss of several potential enhancer sites including ETS, AP-1, STAT, OCT1 and a CAAT box. In contrast, the *Sca*I fragment was as active as the F-G construct, indicating the presence of repressor elements in the region between the *Dra*I and *Sca*I sites (-8807/-8644), potentially another OCT1 or estrogen receptor half-site (ER). No statistically significant difference in activity was observed between the *Bst*EII, *Dra*I and *Sca*I fragments but the trend of the *Sca*I fragment to have promoter activity equivalent to the full-length construct was consistent. The *Bsu*36I fragment possessed only half the luciferase activity of the full-length construct, suggesting that the *Bsu*36I deletion removed sites for positive regulators of *ELF5* such as the multiple predicted delta crystallin enhancer binding factor 1 (deltaEF1) and upstream stimulatory factor (USF) sites.

The activity induced by the *Nde*I fragment, which deletes 906 bp of the original 1223 bp of the full-length fragment, was also significantly lower than that produced by the full-length fragment. However only a small difference was seen in the activity produced by the *Bsu*36I fragment and the *Nde*I fragment, suggesting that if anything, it is a negative regulatory region that lies between these sites.

*Nde*I, the shortest deletion fragment, contains only 193 bp of DNA 5′ of exon 1. It still retains a significant amount of promoter activity ($p<0.001$) above baseline, indicating that the minimal promoter lies 3′ to the *Nde*I site. To delineate the 3′ end of the *ELF5*

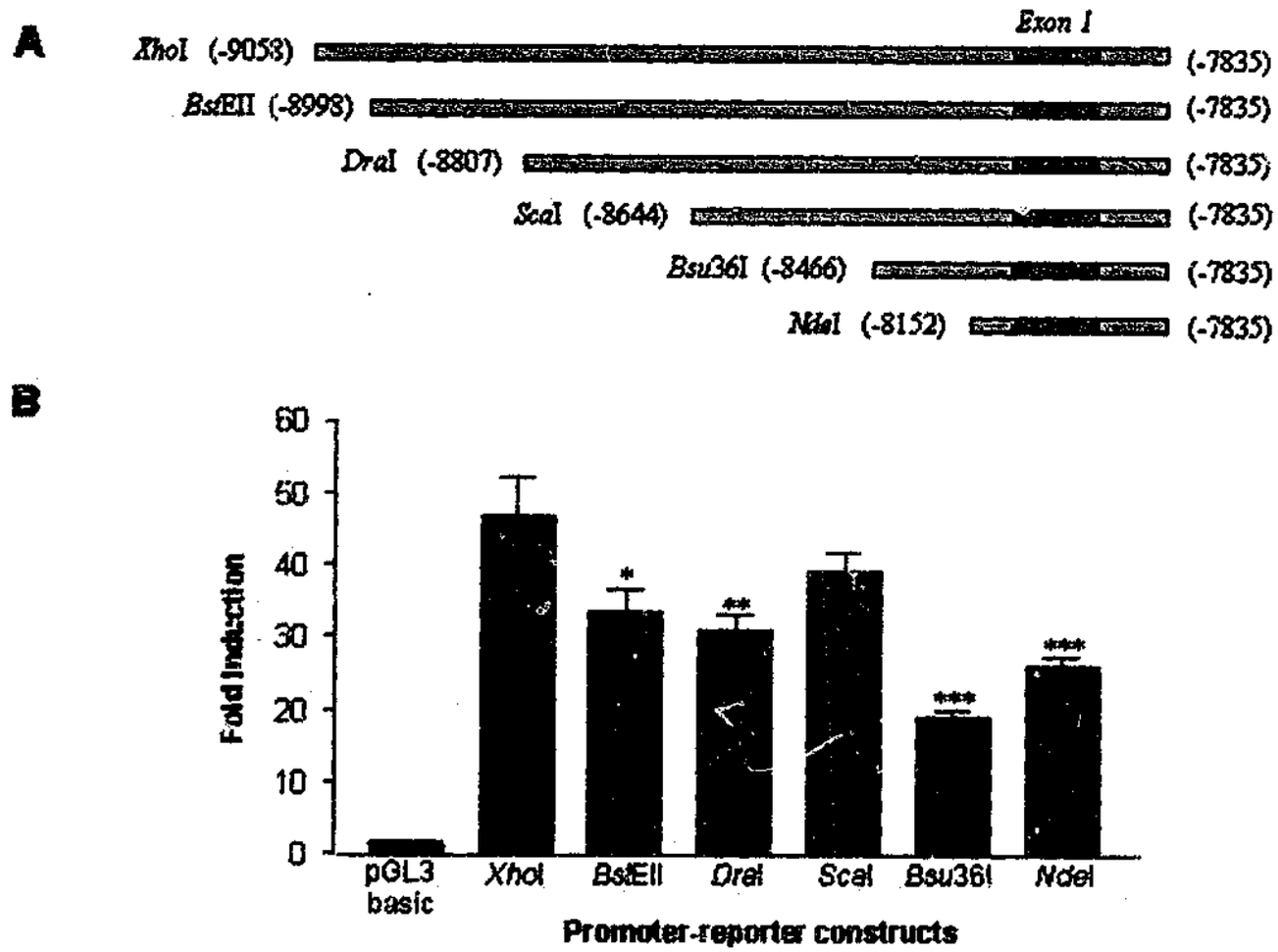


Figure 4.4. Various 5′ end deletions of the functional promoter fragment (A) result in different levels of luciferase activity (B). This indicates the presence of enhancer and repressor elements in the deleted regions. *p<0.05; **p<0.01; ***p<0.001 (One-way ANOVA) represents significant difference in luciferase activity compared to the full-length *Xho*I construct (+/-SEM). Fold-induction is relative to activity of the promoterless pGL3-basic vector. This figure was adapted from the Honours thesis (2002) of Renée Chehab.

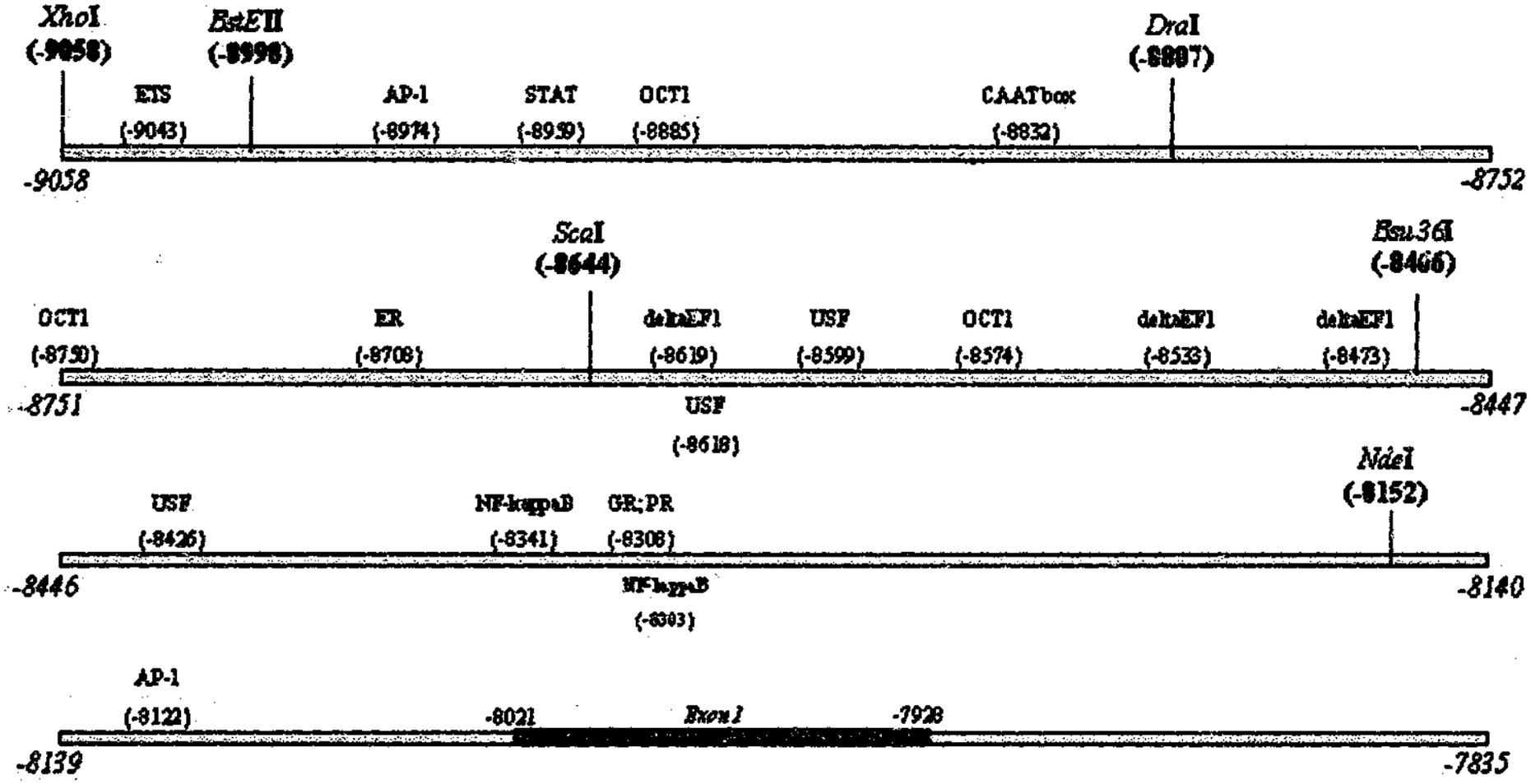


Figure 4.5. Potentially interesting putative binding sites predicted by searches of BCM Search Launcher and MatInspector (see Appendix III for full list of predicted transcription factor binding sites). ETS – ETS factor binding site; AP1 – Activator protein 1; OCT1 – Octamer binding factor 1; STAT – Signal transducer and activator of transcription; ER – Estrogen receptor; USF – Upstream stimulatory factor; deltaEF1 – delta crystallin enhancer binding protein; NF-kappaB – Nuclear factor kappa B; GR – glucocorticoid receptor; PR – progesterone receptor. Adapted from R. Chehab (Honours Thesis, 2002).

regulatory region, a further series of 5′ end deletions were constructed in pGL3, starting at the *Nde*I site and deleting into intron 1. Fragment I-K (Figure 4.1) was used to generate these constructs. Upon deletion of the 32 bp 3′ to the *Nde*I site, promoter activity was ablated (R. Chehab, data not shown). In the initial EGFP reporter assay, the F-G fragment that included 1 kb 5′ region and exon 1 possessed promoter activity, but the F-H fragment encompassing the same 5′ end but lacking exon 1 was not active (Figures 4.1 and 4.3). Therefore, the 161 bp window 5′ to and including exon 1, is necessary for promoter activity, but intron 1 does not possess functional promoter elements. However, the luciferase assay is considerably more sensitive and demonstrates conclusively the lack of promoter activity contained in DNA 3′ to exon 1.

Computer alignment of the human and murine genomic sequences surrounding exon 1 demonstrates a highly conserved genomic region consonant with the smallest active fragment, *Nde*I (Figure 4.6). This indicates the presence of a conserved minimal promoter, while more distant sequence has diverged considerably and likely contributes to differential regulation of the gene between species.

4.3.4 Candidate regulators of the h*ELF5* promoter

Based upon deletion analysis and computer analysis of the functional *ELF5* promoter region as described above, we predicted several candidate regulators of the promoter, paying particular interest to proteins implicated in epithelial carcinogenesis. All experiments were performed in both MCF7 and T47D cells (with the exception of the addition of *ELF5*, which was performed in MCF7 cells only), but for simplicity the data from the T47D cell line is shown. While T47D cells express *ELF5* endogenously,

MCF7 cells do not. However, equivalent fold inductions were obtained from both cell lines, though base-line activity of the promoter was consistently higher in the T47D cell line (data not shown). Using the 1 kb promoter + exon 1 (Fragment F-G, Figure 4.1) region in the luciferase reporter vector, *ELF5, ELF5, AP-1, NFκB* and *STATs 1, 3, 5a* and *5b* were tested as candidate regulators of the promoter. The *ELF5* promoter possesses one *ets* site (-9043), the sequence of which is compatible with binding by *ELF5* (Oettgen et al., 1999b), and therefore is a potential site for autoregulation. The *ELF5* promoter reporter construct was transiently tranfected into three sub-lines of MCF7 cells: a derivative of the parental line, which does not express *ELF5*, and two other derivatives that stably express the p30 and p20 isoforms of *ELF5* respectively. Presence of p30 did not significantly alter promoter activity, but p20 repressed the promoter (Figure 4.6A). This indicates that while full-length *ELF5* does not act on its own promoter, production of the truncated isoform can act as a negative feedback system to attenuate further *ELF5* transcription. Therefore, another *ets* factor must act through the EBS, if indeed it is a functional site. This experiment was not performed in T47D cells, as an *ELF5*-null derivative of this line is not available.

The closely related *ets* factor, *ELF3*, which is also expressed in the mammary gland but is transcriptionally downregulated when *ELF5* levels increase and vice versa, did not have any effect on *ELF5* promoter activity in MCF7 or T47D cells (Figure 4.7D).

Two putative NFκB response elements were predicted in the *ELF5* promoter at –8341 and –8303. *NFκB* was transfected into MCF7 and T47D cells in three different molar ratios compared to the amount of *ELF5* promoter-reporter construct. At amounts of 4-fold less than the reporter vector, 2-fold less and then at an equimolar concentration,

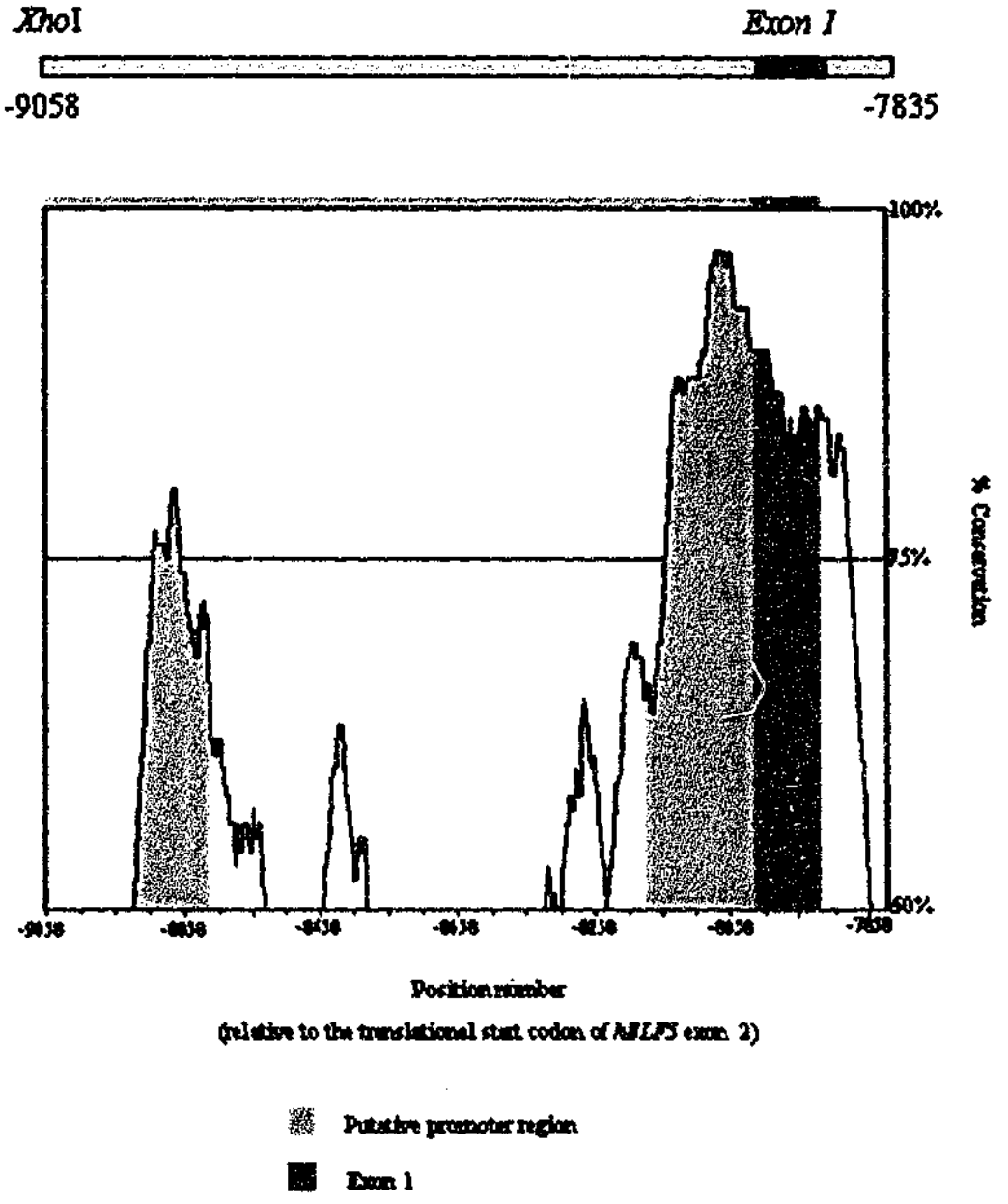


Figure 4.6. VISTA alignment of the murine and human *ELF5* putative promoter sequences. This figure was adapted from the Honours thesis of Renée Chchab (2002).

NFκB repressed *ELF5* promoter activity in a dose-dependent fashion in T47D (Figure 4.7C) and MCF7 cells.

STAT 1, 3, 5a and 5b, which are all relevant to the development of the mammary gland, are putative regulators of the promoter through a predicted site at –8959. Expression plasmids were transfected into T47D cells in 2-fold less (data not shown), and in equimolar concentration relative to the *ELF5* promoter reporter vector (Figure 4.7B). Following stimulation with prolactin, the presence of STAT1 doubled the level of transcription from the *ELF5* promoter ($p>0.01$). STATs 3, 5a and 5b did not significantly affect luciferase activity, indicating that these proteins did not act on the promoter in this assay.

4.4 Discussion

4.4.1 The human and murine *ELF5* genes are not regulated identically

Elucidation of the genomic structure of human *ELF5* revealed alterations in the sequence and structure of the regulatory regions between the mouse and human loci. These changes may provide explanations for different regulation of the gene between human and mouse species. While two alternative transcripts of *Elf5* have been identified in mouse, these differ by alternative 3′ UTRs, which may affect transcript stability (Zhou et al., 1998). Three alternatively spliced transcripts have been identified in human, but these utilise the same 3′ UTR whilst differing in the coding region. The main transcript, p30, is equivalent in the coding sequence to murine *Elf5*. One isoform (ESE-2a) adds ten amino acid residues to the N-terminus of the protein by using the alternative exon 1A in place of exon 1 (Oettgen et al., 1999b). This exon and its

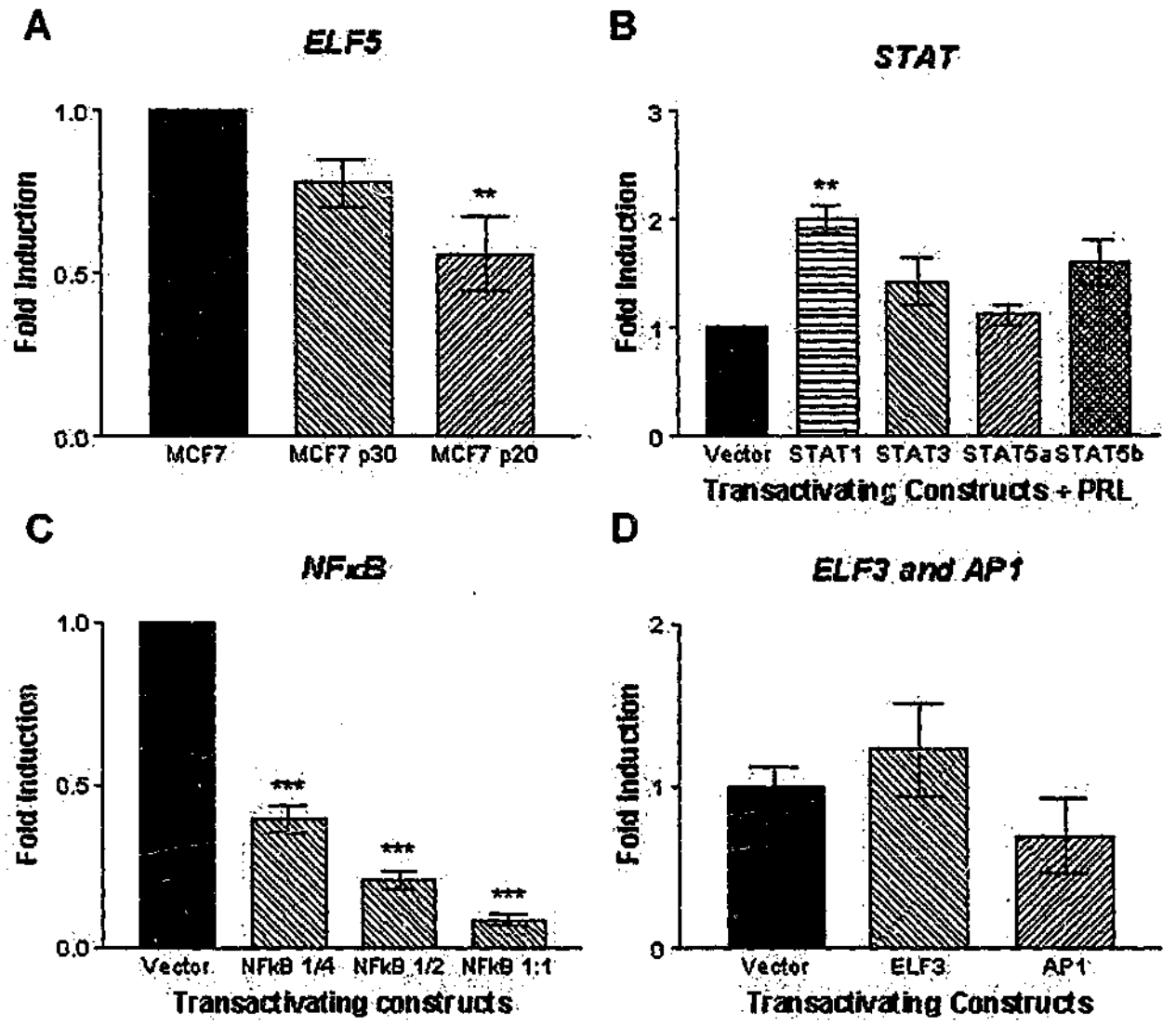


Figure 4.7. Analysis of the effect of various transcription factors on *ELF5* promoter activity. *ELF5* does not significantly affect its own promoter activity in MCF7 cells expressing full-length *ELF5*, but p20 represses transcription by ~40% ($p<0.01$) (A). In T47D cells, of the STAT proteins examined, only STAT1 significantly affects luciferase activity (B). *NF^{K}B* represses activity of the *ELF5* promoter in a dose-dependent manner in T47D cells (C). Neither *ELF3* nor *AP1* significantly alter luciferase activity generated by the *ELF5* promoter luciferase construct in T47D cells (D). n>9. Error bars: +/- SEM. ** $p<0.01$; ***$p<0.001$ (One-way ANOVA).

resultant transcript have not been identified in the mouse. A second isoform is a truncated version of p30, splicing out exons 3-5 that code for the *pointed* domain (Chapter 5). The presence of a single nucleotide divergence from the consensus within the exon 3 5′ splice donor site may contribute to the ability of this exon to be spliced from the human transcript, although the murine locus contains the same change (Genbank Accession AL954374, position 107380). This indicates that another regulatory alteration bears the primary responsibility for splicing out exon 3 in the human, but not the mouse, gene. Therefore, despite the 98% amino acid similarity between murine and human ELF5 proteins, interspecies divergence in regulation of the gene is evident.

Concordantly, alignment analysis of the human and mouse 5′ genomic regions indicates only one area of high homology, closely associated with the conventional exon 1. This region encompasses the minimal promoter region identified in our study (R. Chehab, Honours Thesis, 2002) and accounts for basal transcription of *ELF5*, and possibly for tissue-specific expression as this utility is conserved between species. However, outside of the minimal promoter region the species' sequence diverges, probably accounting for differences in regulation of transcriptional isoforms between species. The alternative exon 1A is not identified as a region of significant homology between mouse and human loci in VISTA.

4.4.2 Predicted regulators of the h*ELF5* promoter

The portion of the promoter region that is not highly conserved between mouse and human species clearly has a functional role in promoting transcription of the gene.

Progressive deletion of the 1 kb region 5' to exon 1 results in a statistically significant progressive decrease in transcriptional activity, with the exception of the mid-length *Sca*I fragment that has wild-type activity, indicating the presence of gene-specific enhancer elements. Some predicted response elements are of vital interest when considering the cellular contexts in which *ELF5* is found. These include OCT1 sites. The OCT1 transcription factor is a POU homoebox family member and its expression is regulated during pregnancy-associated mammary gland development (Malewski et al., 2002), where among other functions, it transcriptionally regulates the milk protein, β-casein (Zhao et al., 2002). β-casein is co-regulated by *ELF5* (Harris, unpublished data) and *STAT5a* (Beaton et al., 2003). OCT1 is inactivated during involution (Marti et al., 1994), concurrently with the downregulation of *ELF5* transcript levels, making it a relevant candidate regulator of *ELF5* transcription. Likewise, the estrogen-responsive zinc-finger homeodomain transcription factor deltaEF1 is a candidate for regulating *ELF5*. *ELF5* does not appear to be directly estrogen-responsive (Chapter 5) but is upregulated during the estrogen-dependent mammary gland development (Zhou et al., 1998), necessitating an intermediary between estrogen signalling and transcription of the gene. USF can act in delta-EF1-containing complexes (Dillner and Sanders, 2002) to regulate secondary estrogen-response genes. The clustering of delta-EF1 and USF sites on the *ELF5* promoter is provocative for the hypothesis that *ELF5* is part of the secondary, rather than the primary, estrogen response.

Comprehensive testing of potentially relevant regulators of the region marked on Figure 4.5 is beyond the scope of this study, but some proteins of interest were tested functionally in luciferase assays to determine whether they had activity on the promoter. Equivalent results were obtained in two breast cancer-derived cell lines, the *ELF5*-

expressing T47D and the *ELF5*-null MCF7. Both cell lines are estrogen receptor positive, an important point considering that *ELF5* is potentially regulated by estrogen signalling in the mammary gland (Zhou et al., 1998). Clearly, T47D possesses the factors required for transcription of *ELF5*, but in MCF7 cells that is not certain. Both cell lines expressed the promoter construct, but at a higher relative level in T47D. Therefore, inactivation of *ELF5* in MCF7 is probably due not to the absence or mutation of a critical upstream factor, but to changes in the *ELF5* genomic locus. These may include silencing of the gene associated with hypermethylation and an inactive chromatin state, or to LOH. These are both common phenomena on the 11p13 region (Fabre et al., 1999; Gudmundsson et al., 1995; Winqvist et al., 1995).

4.4.3 *Ets* regulation of the *ELF5* promoter

ELF5 itself was tested as a potential autoregulator of its own promoter given the presence of a site in the promoter that is technically compatible with *ELF5* binding (Oettgen et al., 1999b). However, full length *ELF5* had no effect on reporter gene activity, indicating that another *ets* factor or factors may act through this site. p20 repressed promoter activity, indicating that in the context of *ELF5* transcription, it may be a dominant negative inhibitor of expression. p20 does not seem to be a highly expressed isoform in normal tissue (unpublished data), making the possibility of an autoinhibitory feedback loop a less likely scenario. This particular *ets* motif is also compatible with *ELF3* binding. *ELF3* has a highly similar flanking nucleotide preference to *ELF5* (Oettgen et al., 1999b), and only one of the flanking nucleotides on this motif is preferential for *ELF3* binding over *ELF5*. Co-transfection of the *ELF3* expression plasmid in combination with the *ELF5* promoter-luciferase reporter construct showed no significant transactivation or repression of promoter activity. We

had hypothesised that *ELF3* may transcriptionally repress the high level of *ELF5* transcription that exists in the lactating mammary gland, acting as part of a switch in the genetic programme that alters the state of the glandular epithelium from proliferation, differentiation and maintenance to apoptosis and involution. However, this role must be taken by an alternative transcription factor, possibly *ESE-3* or a non-*ets* factor.

4.4.4 NFκB regulation of the *ELF5* promoter

Secondly, NFκB was selected as an interesting candidate regulator of the promoter. NFκB is over-expressed or activated in breast and prostate carcinomas, among other cancer types (reviewed in Rayet and Gelinas, 1999). It commonly interacts with ets proteins on target promoters (reviewed in Li et al., 2000), although the literature only comments once on whether it alters transcription of *ets* genes themselves. In this case, following interleukin-1β (IL-1β) stimulation, NFκB transactivates the *ELF3* promoter to a high degree in cells such as synovial fibroblasts, chondrocytes, osteoblasts, and monocytes (Grall et al., 2003).

NFκB can act as either an activator (Espinosa et al., 2002; Wu et al., 1997; Wu et al., 2000) or a repressor (Jewett et al., 2003; Morishima et al., 2003) of gene transcription depending on the cellular and promoter context. The presence of putative NFκB response elements between *Bsu*36I and *Nde*I sites correlates with a small rise in promoter activity after deletion of these elements, intimating that they are responsible for promoter repression. This correlates well with the results of the reporter assays where co-transfection of varying molar ratios of NFκB results in dose-dependent promoter repression. While we have not identified which of the two predicted elements

NFκB acts through, further studies using the yeast-one-hybrid system, or mutation analysis of sites, will be capable of identifying the relevant site. These data imply that one of the effects of inappropriate activation of NFκB in cancer may be to directly repress *ELF5* expression, providing one explanation for the loss of *ELF5* mRNA in some cancers and cancer cell lines (Oettgen et al., 1999b; Zhou et al., 1998).

4.4.5 STAT regulation of the *ELF5* promoter

The *ELF5* promoter region also possesses one predicted STAT binding site, at position –8959. The ubiquitously expressed STATs, 1, 3 and 5, are ideal candidates for regulating a cancer and mammary development associated gene, whereas STATs 2, 4 and 6 have specific roles in the immune response. STATs 1, 3 and 5 are signal transducers that produce a growth-promoting response to a wide variety of cytokines and growth factors, and are in some cases even activated simultaneously by the same ligand (reviewed in Bromberg, 2000). They all have functions in the mammary gland, particularly STATs 3 and 5, which are crucial regulators of normal mammopoiesis and lactogenesis (Teglund et al., 1998; Udy et al., 1997) and are and are activated in cancerous mammary epithelium (Watson, 2001). The *Stat5a* null mouse displays a mammary gland phenotype almost identical to that of the *Elf5* heterozygote: the curtailing of lobuloalveolar outgrowth during pregnancy and then failure of lactation post-parturition (Liu et al., 1997b). *Stat5a* and *Elf5* are critical mediators of prolactin receptor signalling to milk protein gene expression (Harris et al., submitted) and *Stat5a* activation increases greatly at d14 pregnancy (reviewed in Watson and Burdon, 1996), about the same time that *Elf5* is dramatically upregulated (Zhou et al., 1998). *Stat5b* null mice, to a lesser extent, have defects in mammopoiesis and lactogenesis (Teglund et al., 1998; Udy et al., 1997). *Stat3* also has a role in the mammary gland – a

paradoxical role considering the promotion of growth and resistance to apoptosis that it confers on cancerous cells (Bromberg, 2000; Bromberg et al., 1998; Calo et al., 2003). Conditional mutation of *Stat3* in the mammary gland reveals that it is required in mammary involution, where epithelial cells apoptose and the gland returns to a pre-pregnant state (Chapman et al., 1999).

STAT1 is involved in mammary gland development and integrates interferon (IFN) signalling (Ramana et al., 2002). STAT1 null mice display specific defects in the IFN response (reviewed in Akira, 1999). Although increased levels of STAT1 have been observed in a wide variety of human cancer types, the protein is a potential tumour suppressor as it is important for cell cycle arrest and initiation of apoptosis in response to IFN signalling (Calo et al., 2003). High levels of STAT1 activation in breast cancer are predictive of a longer relapse-free survival (Widschwendter et al., 2002).

Conversely, STAT3 possesses oncogenic properties. It is constitutively activated by Src and JAK kinases in breast cancers and is required for v-src-mediated transformation (Bromberg et al., 1998; Garcia et al., 2001). It is activated by BRCA1 in prostate cancers (Gao et al., 2001); and is constitutively active in ovarian carcinoma (Huang et al., 2000). STATs 3 and 5 are constitutively activated in nasopharyngeal cancer (Hsiao et al., 2003). STAT5b is constitutively activated in ER positive breast cancers and the presence of a dominant-negative STAT5b results in apoptosis in breast cancer cell lines (Yamashita and Iwase, 2002; Yamashita et al., 2003), reiterating the dependence of the cancerous phenotype on the anti-apoptotic and growth-promoting properties of constitutive STAT activation. Likewise, a number of STAT 3 and 5 target genes are established as affecting breast cancer progression or prognosis. These include *cyclin D1*

(Matsumura et al., 1999; Weinstat-Saslow et al., 1995), *c-myc* (Kiuchi et al., 1999) and *Bcl-X_L* (Socolovsky et al., 1999).

In the STAT-*ELF5* promoter luciferase assays, T47D cells were exogenously stimulated with prolactin to imitate the state that murine mammary glands undergo when they express *Elf5* at the highest level during pregnancy-associated mammary gland development. This stimulation should provide the cellular context required for transcription factors to be active on the *ELF5* promoter. STAT1 activation led to a small but significant ($p>0.01$) increase in *ELF5* promoter activity. While STATs 3, 5a and 5b tended to increase *ELF5* promoter activity, this was neither a consistent nor a significant result. Particularly STAT5a, the most likely candidate of the STATs for an *ELF5* regulator due to its participation in the PRL/PRLR pathway along with *ELF5*, did not result in any difference in luciferase activity.

These data indicate that only STAT1 dimers bound to the predicted STAT response element in the *ELF5* promoter. This may be due to the lack of appropriate signalling stimulation in this system that, via the JAKs, modifies the STATs to dimerise, translocate to the nucleus and become transcriptionally active. Although MCF7 and T47D express both prolactin (Shaw-Bruha et al., 1997) and the prolactin receptor, respond to exogenous prolactin by migration along a prolactin gradient (Maus et al., 1999) and activate signalling response pathways (Das and Vonderhaar, 1996), they do not respond by expressing markers of terminal differentiation (STAT-induced milk proteins such as β-casein and whey acidic protein) (Fuh and Wells, 1995). This could be due to a defect near this endpoint of the pathway, in one or more of the protein interactions that *ELF5* is likely to be involved in. Alternatively, the predicted STAT

response element in the promoter may only be bound by STAT1 because of the physical structure of the promoter, and the necessity for specific protein interactions between transcription factors, that results in promoter activation. The association of STAT1 as a growth inhibitor and inducer of apoptosis complements the predicted function of *ELF5* as an epithelial tumour suppressor. STAT1 transactivation of *ELF5* may be one of the mechanisms by which STAT signalling inhibits cell growth and survival.

4.4.6 Conclusions

Confirmation of the relevance of these candidate regulators of the *ELF5* promoter will require further study. The specificity of the interaction with DNA will be determined by site-directed mutagenesis of the putative binding motif and by EMSA. Chromatin immunoprecipitation (ChIP) analysis using input from tissue samples will assist in determining the relevance of the interaction to the *in vivo* context.

Interestingly, only one positive regulator of the promoter has yet been identified. It is possible that alternative assay systems would have a greater advantage in identifying transcriptional enhancers for the *ELF5* promoter. MCF7 and T47D cells are non-metastatic cancerous cells, which are no longer sensitive to a variety of cues that normally signal mammary epithelial cells to proliferate, differentiate into three-dimensional acini, secrete milk and involute as happens in a functional mammary gland. The luciferase assays performed in this study utilised a two-dimensional culture system, lacking three-dimensional cell interactions and exogenous steroid and prolactin stimulation that are present in the pregnant and lactating mammary gland, where *Elf5* transcription is dramatically upregulated. Investigating the transcriptional regulation of *ELF5* in a three-dimensional culture system to better model the *in vivo* situation may

provide a wealth of additional, relevant information in elucidating the factors that impact the regulation of this factor at the mRNA level.

Chapter 5. A novel protein isoform of the epithelial-specific ets transcription factor, *ELF5*, regulates the *BRCA1* promoter in breast cancer cell lines

5.1 Introduction

5.1.1 Oncogenic *ets* transcription factors

Ets transcription factors commonly regulate genes involved in cell proliferation, differentiation, tissue specificity and apoptosis. It is not surprising, therefore, that they have been implicated in various types of cancer. Several *ets* genes including *Ets-1* (Seth and Papas, 1990), *Erg* (Hart et al., 1995), *Tel* (Ritchie et al., 1999), *Fli-1* (Delattre et al., 1992; Gavrilov et al., 2001) and *E1AF* (Habelhah et al., 1999) have been shown to be proto-oncogenes. For example, *in vitro* experiments demonstrate that over-expression of murine Ets-1 in NIH3T3 cells induces colony formation in soft agar and tumour growth in nude mice, and confirm the oncogenic potential of this gene (Seth and Papas, 1990). More recently, aberrant expression of ETS-1 protein in ovarian epithelial carcinoma was found to correlate with higher clinical stage and poor prognosis (Takai et al., 2002).

5.1.2 Tumour suppressor *ets* transcription factors

Other *ets* factors such as ERF have been shown to act as tumour suppressors, reverting ets-related oncogenesis (Sgouras et al., 1995). We postulate that *ELF5* may be another *ets* gene with tumour suppressing capability based on its lack of expression in a large variety of carcinoma cell lines (Oettgen et al., 1999b; Zhou et al., 1998) and in primary breast cancers (Ma et al., 2003a) and on its chromosomal localisation to 11p13, a region that frequently undergoes LOH and hypermethylation in various epithelial-derived neoplasias, particularly those of the breast, prostate and lung (Bepler et al., 1998; Fabre et al., 1999; Gudmundsson et al., 1995; Karnik et al., 1998b; Koo et al., 1999; Ludwig et al., 1991; Shaw and Knowles, 1995; Shipman et al., 1998; Shipman et al., 1993; Tran and Newsham, 1996; Vandamme et al., 1992; Viel et al., 1992; Virmani et al., 1998; Winqvist et al., 1995). *ELF5* is also a member of the ESE subfamily of *ets* genes. The other members of this subfamily, *ELF3* and *ESE-3*, have also been implicated in cancer as a breast-specific oncogene and a tumour suppressor gene, respectively (Eckel et al., 2003; Tugores et al., 2001).

As *ELF5* is a transcription factor, it is likely that downstream target genes are dysregulated if *ELF5* expression is decreased during carcinogenesis. We therefore investigated the possibility that *ELF5* may regulate an epithelial tumour suppressor gene at the transcriptional level. We chose to investigate *BRCA1* given its involvement in suppression of breast tumourigenesis, the fact that it is transcriptionally regulated by *ets* factors (Atlas et al., 2000; Xu et al., 1997b), and based on a distinct phenotypic overlap between mice with reduced *Brca1* and reduced *Elf5* mRNA expression.

5.1.3 Mouse models for *Elf5* and *Brca1* deficiency

Recently an *Elf5* heterozygous mouse line has been generated (Appendix VII). While the null mice demonstrate early fetal lethality, the heterozygous females have a mammary gland phenotype that overlaps with that of the dominant-negative *Brca1* transgenic mouse (Brown et al., 2002). Mammary glands of these mice demonstrate delayed pregnancy-associated mammary gland development and reduced lactation. In the dominant-negative *Brca1* transgenic, mothers show delayed lobuloalveloar development that recovers with subsequent pregnancies. The *Elf5* heterozygotes display a more severe phenotype with minimal lobuloalveolar differentiation and failure of lactation, which does not recover after the first pregnancy. These results indicate that a genetic interaction may occur between *ELF5* and *BRCA1*.

Null mutants for *Brca1* in the mammary gland display a similar phenotype. Mutation of *Brca1* in the mammary epithelium using MMTV-*Cre* to splice out the 'floxed' *Brca1* exon 11 alleles, resulted in impaired mammary development. Mutant glands were underdeveloped in pregnancy and showed increased levels of apoptosis. Unlike the *Elf5* heterozygotes, females could nurse pups. Between ten and 15 months, about a quarter of the animals developed mammary tumours (Deng, 2001). As the phenotype is late-onset and not fully penetrant, the lack of *Brca1* may result in genomic damage and dysregulation of other tumourigenesis-related genes.

5.1.4 Changes in *BRCA1* are found in breast carcinoma

Mutations in the human variant of the tumour suppressor gene *BRCA1* are found in a high percentage of hereditary breast and ovarian cancers (Easton et al., 1993). While defects in the gene have not been observed in sporadic breast cancers (Futreal et al.,

1994), most of these cancers show decreased BRCA1 expression (Thompson et al., 1995), indicating that mechanisms other than mutations in the coding region are responsible. Indeed, promoter hypermethylation with associated gene silencing has been described by various groups (Dobrovic and Simpfendorfer, 1997; Mancini et al., 1998; Rice and Futscher, 2000; Rice et al., 1998; Rice et al., 2000). Downregulation of the gene by the HMGA1b transcription factor, which is over-expressed in sporadic breast cancer, has also been suggested as a reason for the decreased protein levels in carcinoma (Baldassarre et al., 2003). Aberrant cytosine methylation, histone hypoacetylation and chromatin condensation encompassing a critical promoter subregion flanking *BRCA1* exon 1A also correlate with decreased *BRCA1* expression in breast and ovarian cancers (DiNardo et al., 2001; Rice and Futscher, 2000; Rice et al., 1998). This promoter subregion includes the cAMP response element binding site (CREB) linked to an EBS, indicating that silencing of these elements affects *BRCA1* transcription.

5.1.5 Regulation of the *BRCA1* gene

The BRCA1a and BRCA1b protein isoforms have been implicated as protein partners of a variety of *ets* proteins including ELK-1, SAP1, ETS-1, ERG-2 and Fli-1, interacting via the *ets* domain (Chai et al., 2001). ELK-1 co-operation with BRCA1 enhances the growth suppressor capacity of BRCA1 in cultured breast cancer cells, and depresses the transcription of the ELK-1 target *c-fos*, whereas the presence of the closely related SAP-1 protein does not (Chai et al., 2001). This suggests that one of the pathways utilised by BRCA1 in tumour suppression involves an *ets* signal transduction pathway.

The *BRCA1* genomic locus lies head-to-head with the unrelated *NBR2* gene, and a short 218 bp intergenic region lies in between the first exons of either gene. This region has been shown to function as a bi-directional promoter (Xu et al., 1997b). It drives expression of both genes, though to differing levels. BRCA1 is expressed in a restricted set of tissues including breast epithelium, ovary, kidney, placenta, testis and thymus, while NBR2 is expressed more widely (Xu et al., 1997a). This indicates that there are tissue-specific elements acting on the promoter differentially in the two directions even though the minimal defined promoter region acts bi-directionally. One candidate for this interaction is *ELF5*, which is expressed strongly in mammary epithelium and other epithelium-rich organs such as kidney, but not in non-epithelial tissues (Oettgen et al., 1999b; Zhou et al., 1998).

Four consensus *ets* motifs are present in the *BRCA1α* promoter region that flanks exon 1A (Xu et al., 1997b). Two of these are single sites, associated with a CREB site (ets#3) and an AP-1 site (ets#4), and one is a tandem *ets* site (ets#1/2) shown to be bound by the GABPα/β tetramer, a ubiquitously expressed *ets* factor (Figure 5.1). This site appears to be critical for transcription of the gene (Atlas et al., 2000; Boccia et al., 1996).

BRCA1 is regulated by estrogen signalling (Marquis et al., 1995) in a late-induction time course, indicating that regulation is via a cascade of estrogen-response genes and not as a result of estrogen receptor binding to DNA (Gudas et al., 1995; Spillman and Bowcock, 1996; Xu et al., 1997b). *ELF5* is also a potential target of estrogen-induced transcription: it has a predicted ER half-site in its promoter region (Chapter 4) and in the mouse, is dramatically upregulated during the estrogen surge in pregnancy-associated

mammary development. Therefore *ELF5* is also a candidate to regulate *BRCA1* in response to estrogen signalling.

5.1.6 Transcriptional isoforms of *ELF5*

Two distinct transcriptional isoforms of *ELF5* have been isolated. The full-length transcript encodes a 30 kDa protein, whereas an alternatively spliced isoform loses the *pointed* protein interaction domain and encodes a 20 kDa protein. In this chapter we show that the shorter p20 protein isoform transactivates the *BRCA1* promoter in a dose-dependent fashion via direct binding to the most proximal of the *ets*-binding sites. This regulation is not dependent upon steroid hormone regulation. In contrast, p30 is a functional transcription factor, but not in the context of the *BRCA1* promoter.

5.2 Methods

5.2.1 Isolation of the p20 isoform of ELF5

The p20 transcript was isolated by 5′ rapid amplification of cDNA ends (RACE) from human lung cDNA. The Marathon RACE kit (Clontech) was used according to the manufacturer's instructions. This cDNA was isolated by Jiong Zhou in this laboratory.

5.2.2 Construction of *ets* expression plasmids

All restriction endonucleases and DNA-modifying enzymes were purchased from Promega Australia. *ELF5* p30 and p20, *c-fos*, *c-jun* and *ELF3* cDNAs were blunt-ended using Klenow DNA polymerase and blunt cloned into the pEF-BOS expression vector (Mizushima and Nagata, 1990) using the *Xba*I sites. These constructs were made by Ross Thomas.

5.2.3 Site-directed mutagenesis of *BRCA1* promoter pGL3 reporter constructs

The pGL3 luciferase reporter plasmids (Promega) containing the sense and antisense BRCA1α promoter were previously described (Xu et al., 1995). These constructs, and the pGL3-BRCA1-*ets#1/2mt* plasmid, were generously supplied by Dr M. Brown (Dept. Biochemistry, Queensland University). Two plasmids containing mutations in the ets #3 and #4 sites were constructed using PCR-based site-directed mutagenesis. Briefly, the ets #3 mutant was generated by PCR between the 5′ (NBR2) end of the promoter (oligonucleotide 16, Appendix I) and the ets#3 site (oligonucleotide 17) and by a separate PCR between the ets#3 site (oligonucleotide 18) and the BRCA1 end of the promoter (oligonucleotide 19). These products were annealed in an equimolar ratio and used as template for an overlap PCR to generate the full-length promoter fragment containing the mutated site. The ets #4 mutant was generated using oligonucleotide 16 with oligonucleotide 20. PCR conditions were the same for the generation of both mutant constructs and utilised an annealing temperature of 55°C and an extension of 1 minute, with Pfu DNA polymerase (Promega).

5.2.4 Production of the prostate-specific antigen promoter luciferase reporter construct

The PSA promoter (Genbank Accession S81389) was isolated from normal human blood genomic DNA with oligonucleotides 33 and 34 (Appendix I), using the Elongase enzyme mix (GibcoBRL) with an annealing temperature of 55°C and 1 minute extension. The promoter fragment was cloned into the T/A cloning site of pGEMT-Easy (Promega) then subcloned by releasing the fragment with *Nco*I and *Sal*I and inserting into the *Sma*I/*Xho*I sites of pGL3 to generate the reporter construct.

5.2.5 Production of hELF5 promoter pGL3 luciferase reporter constructs

These plasmids were described in Chapter 4.

5.2.6 Transfections and luciferase assays in MCF7, T47D and CV-1 cell lines

MCF7 and T47D human breast ductal carcinoma cell lines and CV-1 green monkey kidney immortalised cell lines were obtained from the American Type Culture Collection (ATCC). MCF7 and T47D cell lines were maintained in RPMI-1640 medium (GibcoBRL) supplemented with 10 % (v/v) FCS (CSL) and kept in a humidified 37°C, 5 % CO_2 incubator. CV-1 cells were maintained in DMEM with 0.1 mM non-essential amino acids (GibcoRL), 1 mM sodium pyruvate (GibcoBRL) and 10 % (v/v) FCS. Cells were plated for transfection in 24-well plates (Becton-Dickinson) at a density of $5x10^4$ cells per well and incubated overnight. A total of 0.5 µg DNA was transfected per well, comprising 83 ng pSV-β-galactosidase plasmid (Promega) as a transfection efficiency control, with the remaining DNA made up of 50 % molar ratio of

the relevant pGL3 reporter construct and an equimolar amount of pEF-BOS constructs. DNA was prepared using the Qiagen Endo-Free maxiprep kit (Qiagen). Transfections were carried out using FuGene 6 transfection reagent (Roche Molecular Biochemicals) and incubated for 48 hours. Where appropriate, 17β-estradiol or progesterone was added to transfected cells at concentrations of 10 nM and 10 μM respectively. Cells were harvested using the Constant Light Signal Luciferase kit (Roche). Luciferase activity was measured on the Lumicount (Hewlett-Packard) then 50 μl of lysate was incubated with an equal volume of 2 X β-galactosidase sample buffer (4.5 mM 2-nitrophenyl-β-D-galactopyranoside, 0.08 % (v/v) β-mercaptoethanol, 2 mM $MgCl_2$, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4) at 37°C until colour had developed. Absorption was measured at 415 nm on the BioRad 3550 plate reader. Results were normalised by dividing luminescence units by the β-galactosidase assay readout. All transfection experiments were performed at least three times in triplicate, so that n>9. Results were analysed using the one-way ANOVA and the Bonferroni post-test to compare selected columns (GraphPad Prism Version 2.0). Where $p \leq 0.05$, column values were taken to be significantly different.

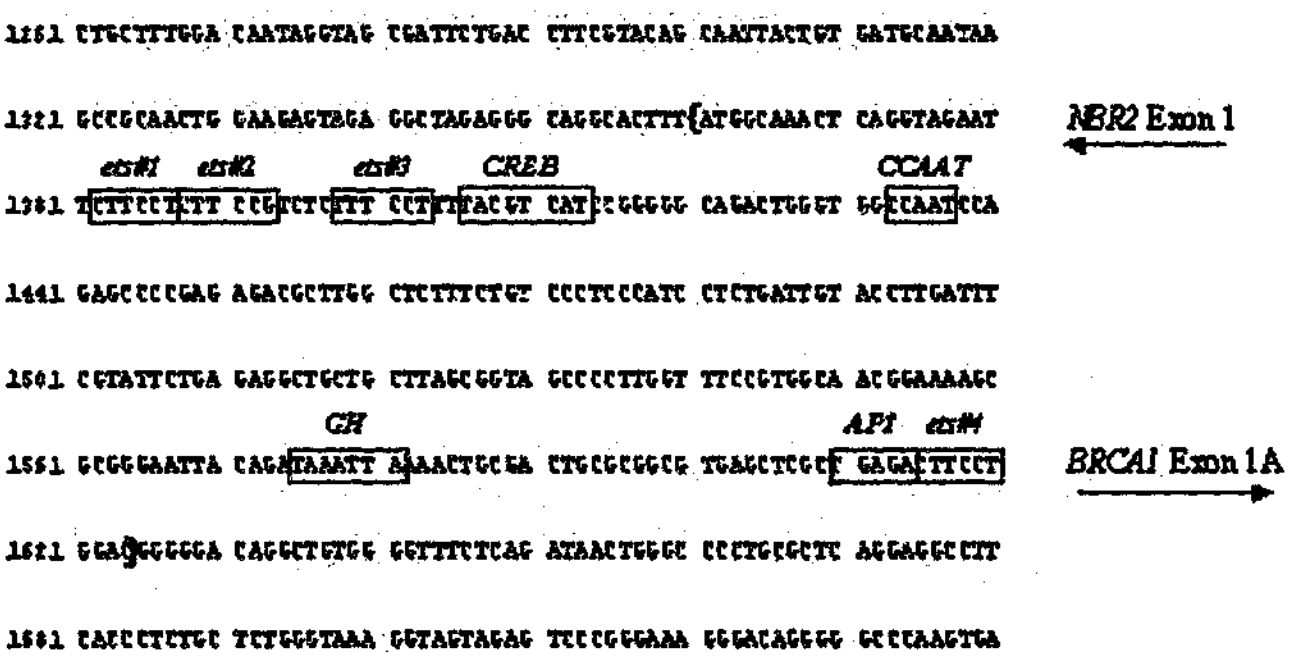


Figure 5.1. Schematic diagram of *BRCA1a* promoter showing potential transcription factor binding sites. The nucleotide position number on the left corresponds to that found in Genbank number U37574 (Xu et al., 1995) sequence. Exons are marked in red lettering. The promoter fragment inserted into the pGL3 vector is in parentheses. Adapted from Xu et al. (1997).

5.2.7 Preparation of Δ33 h*ELF5* constructs

Plasmids encoding *ELF5* recombinant proteins with an N-terminal 33 amino acid deletion were constructed using the pET-21a+ vector (Clontech). The insert was amplified by PCR with oligonucleotides 35 and 36 (Appendix I), using p30 and p20 cDNA templates. The resultant cDNAs were digested with *Bam*HI and *Sac*I and inserted into the *Bam*HI and *Sac*I sites of the pET21a+ vector. Recombinant proteins were expressed from the constructs in BL21(DE3)pLysS bacteria (Clontech) according to the manufacturer's instructions.

5.2.8 Electrophoretic Mobility Shift Assays

Double-stranded oligonucleotides containing the ets #3 and #4 sites in wild-type and mutant form (oligonucleotides 21-28, Appendix I) were used to determine the binding capability of ELF5 recombinant protein to these sites. The E74 consensus *ets* binding motif was used as a positive control for binding of both recombinant proteins (oligonucleotides 29-32). Only the forward sequence is shown; reverse primers were complementary to these.

Electrophoretic mobility shift assays (EMSA) were performed as previously described by (Dignam et al., 1983). Briefly, 200 ng double-stranded oligonucleotides were labelled with [γ-^{32}P] dATP by T4 polynucleotide kinase and purified by running on a 6 % (w/v) native polyacrylamide gel. The labelled double-stranded oligonucleotides were then excised from the gel, eluted in 0.3 M NaOAc (pH 5.2) and ethanol precipitated with 20 μg glycogen (Roche). Reaction mixture was added in the order of H_2O, 5 X

EMSA binding buffer (final concentration 10 mM HEPES (pH 7.8); 2 mM $MgCl_2$; 40 mM KCl; 5 % (v/v) glycerol; 1 mM dithiothreitol (DTT); 1 μg poly(dI.dC).poly(dI.dC), 200 ng recombinant protein, and an 100-fold molar excess of unlabeled competitor oligonucleotide or 0.5 μg anti-ELF5 antibody in a total volume of 20 μl. Reactions were incubated at room temperature for 15 min, then 50,000 counts per minute (cpm) of labelled oligonucleotide probe was added, followed by a further 15 min incubation. Reactions were run on 6 % (w/v) native polyacrylamide gel for 15 cm at 125 V, then dried under vacuum onto Whatman paper and exposed to Kodak Biomax MR film (Kodak) at –80°C.

5.3 Results

5.3.1 Isolation of p20

The p20 *ELF5* isoform was cloned from a λ phage human cDNA lung library by Jiong Zhou (PhD Thesis, 2001). The full-length p30 isoform of human *ELF5* had previously been isolated, as described in (Zhou et al., 1998). This isoform splices out exons 3 to 5, which does not result in a frame-shift but deletes most of the *pointed* domain (Figure 5.2).

5.3.2 h*ELF5* p20 transactivates the BRCA1α promoter

The *BRCA1α* promoter, which contains four potential *ets* motifs, can be transactivated by a truncated isoform of *ELF5*. The p20 isoform, which splices out exons 3-5 of the gene encoding the *pointed* protein-protein interaction domain (Figure 5.1), transactivates the BRCA1α promoter about 2.7-fold over its basal activity in both

MCF7 and T47D cells (Figure 5.3A). As results were equivalent in these two cell lines, representative data from the T47D cells only has been shown. Reporter activity was increased two-fold from this promoter when twice the amount of p20-pEF-BOS expression construct was included in the cell transfections, indicating that transactivation of the *BRCA1α* promoter by p20 is dose-dependent. Notably, the 218 bp *BRCA1α* promoter is a bi-directional promoter that drives the expression of both *BRCA1* and the more widely expressed *NBR2* gene (Xu et al., 1997b). When the promoter was cloned in the anti-sense orientation in the pGL3 vector, p20 also drove expression to the same extent in the *NBR2* direction, indicating that p20-induced transcriptional activity is not unidirectional and may regulate both genes (Figure 5.3B) in tissues where *ELF5* p20 is present.

In many cases, *ets* transcription factors interact with AP-1 on promoters and synergistically transactivate genes. A proportion of *ets* motifs are found in conjunction with AP-1 sites, as is the case with the ets #4 site in the *BRCA1α* promoter (Figure 5.1).

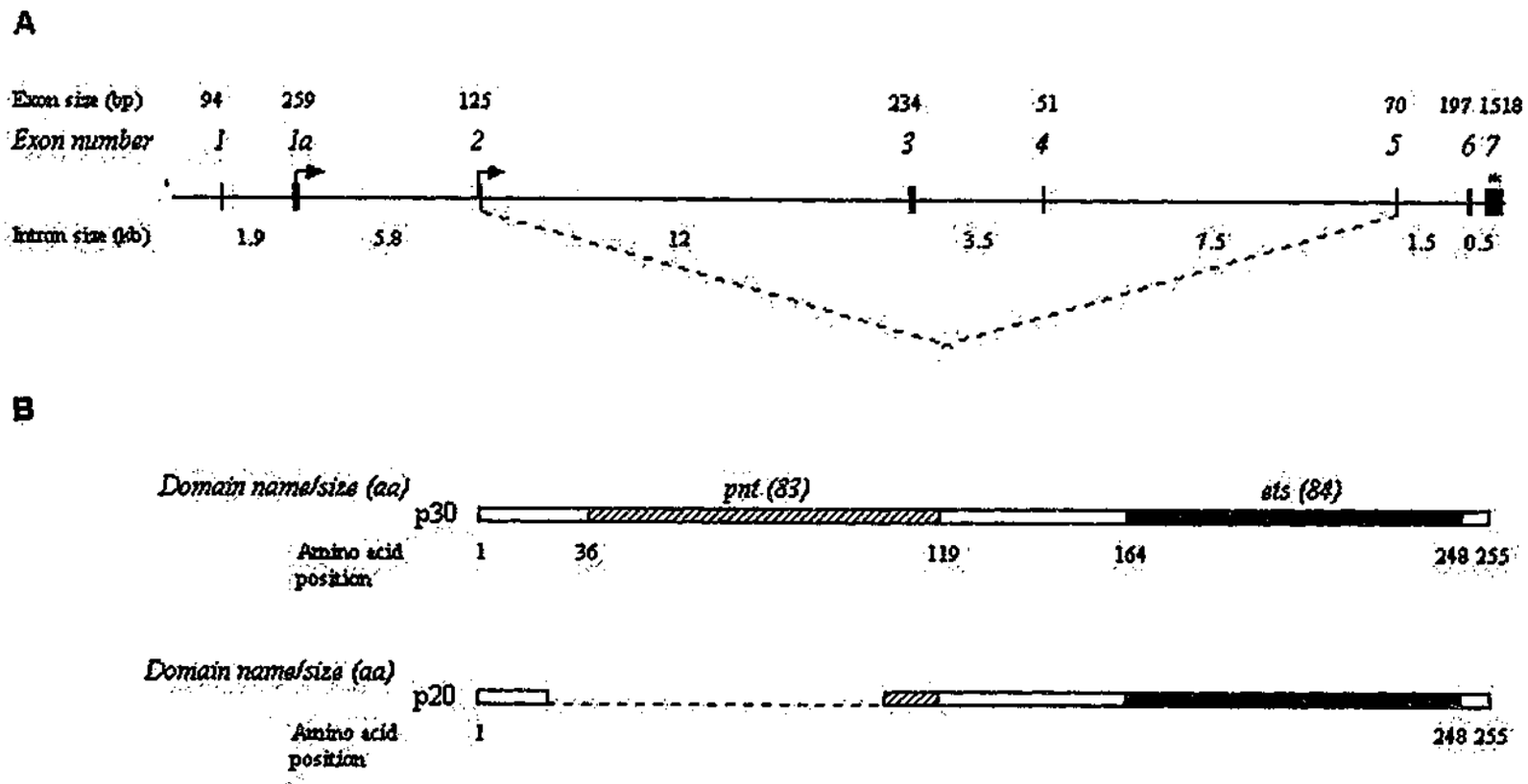


Figure 5.2. Genomic structure of *ELF5* and alternatively spliced isoforms. Genomic structure of the *ELF5* locus, showing the alternative splicing of exons 3-5 that creates the p20 mRNA (A). The protein products resulting from the alternatively spliced transcripts, p30 and p20, are also shown (B). Genomic sequence can be accessed at Genbank accession A137224, p30 protein sequence at Genbank accession NP_001413, whereas the p20 protein sequence has not yet been published.

However, inclusion of the c-fos and c-jun subunits that dimerise to form the AP-1 transcription factor along with p20 in these transfections, failed to show synergistic activity, or indeed any alteration of the ability of p20 to transactivate this promoter (Figure 5.4).

5.3.3 *ELF3* and full-length *ELF5* do not transactivate *BRCA1*

Likewise, *ELF3*, an *ets* transcription factor closely related to *ELF5*, also failed to transactivate the *BRCA1α* promoter, demonstrating that the ability to act on the *BRCA1* promoter is not shared indiscriminately among the epithelial-specific *ets* genes (Figure 5.5). Unexpectedly, the full-length *ELF5* isoform, p30, failed to transactivate the *BRCA1* promoter (Figure 5.3). However, this transcription factor is functional in other promoter and cellular contexts, as it transactivates the *PSA* promoter in CV-1 cells (Figure 5.6).

5.3.4 p20 transactivates *BRCA1* via EBS#4

Site-directed mutagenesis of the *ets* sites in the *BRCA1* promoter demonstrated that p20 acted through the fourth, proximal, AP-1-associated site located in the non-coding *BRCA1* exon 1A, but not through the other sites (Figure 5.7). However, ablation of each site in turn decreased the absolute level of promoter activity compared to the intact *BRCA1α* promoter (data not shown), indicating that these sites are functional and may be bound by other *ets* factors. EMSA performed using *ELF5* Δ33 p30 and p20 recombinant protein on oligonucleotides containing the BRCA1 *ets* #3 and #4 sites,

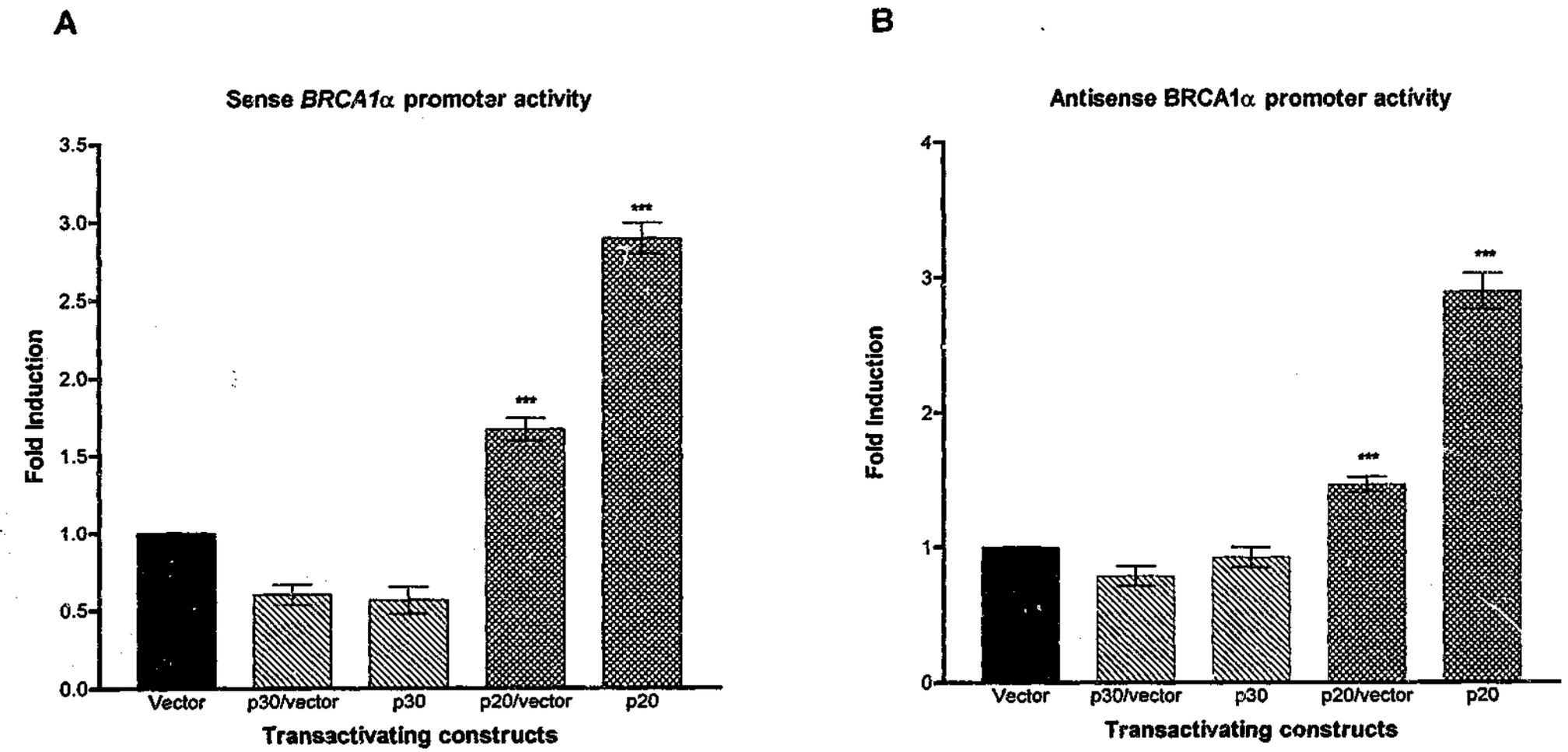


Figure 5.3. ***ELF5* p20 transactivates the BRCA1$^{\alpha}$ promoter.** Transfection of a 1:1 mixture of empty vector and p20 construct (p20/vector) increased luciferase activity of the sense *BRCA1*$^{\alpha}$ promoter almost 2-fold, while transfection of p20 construct undiluted with empty vector (p20) increased luciferase activity 3-fold (A). p20 transactivation of the promoter is therefore dose-dependent. p20 transactivated the promoter in the antisense direction (NBR2 promoter) to the same degree (B). Co-transfection of p30 with the BRCA1$^{\alpha}$ promoter constructs had no significant effect on luciferase activity. Activity of the promoter construct when co-transfected with empty pEF-BOS vector has been arbitrarily set as 1. $n \geq 9$. ***$p<0.001$. Results from transfections into T47D cells are shown.

showed direct shifted bands when the p20 isoform was incubated with the ets #4 site only (Figure 5.8A). This binding was competed by cold oligonucleotide but not by the equivalent oligonucleotide containing a mutation in the *ets* binding site. Band intensity was also decreased by the inclusion of ELF5 antibody, which is directed against a peptide in the DNA-binding domain and therefore would predictably interfere with DNA binding. Consistent with the luciferase assay data, p20 did not bind to the mutated ets #4 site, or to the ets #3 site. p30 did not bind to any of the oligonucleotides derived from the *BRCA1α* promoter (Figure 5.8B), although both ELF5 isoforms interact with the consensus E74 binding site (Figure 5.8C). The N-terminal Δ33 proteins were constructed for the *in vitro* assays as full-length ELF5 p30 and p20 isoforms do not bind specifically to DNA *in vitro* (Oettgen et al., 1999b). This indicates the presence of an N-terminal autoinhibitory domain that requires modification by an as yet unknown cellular process before the transcription factor can be active.

5.3.5 *ELF5* does not transactivate *BRCA1* in direct response to estrogen signalling

Xu et al. (1997b), among others, proposed that *BRCA1α* transcription is upregulated by genes that are under the control of ER binding. The proteins that transduce this signal have not yet been characterised (Spillman and Bowcock, 1996). Therefore we tested the *ELF5* promoter for estrogen-responsiveness, given that there is an ER half-site predicted by computer promoter analysis (TESS; Baylor College of Medicine; Chapter 4). *ELF5* and *BRCA1* transcripts both increase during pregnancy-associated mammary gland development, which is under hormonal control. It is conceivable that estrogen-induced ELF5 protein may upregulate BRCA1 in the mammary epithelial cells. However, the *ELF5* promoter does not appear to be estrogen responsive. Luciferase

activity was neither significantly increased nor decreased after T47D cells, which express both estrogen and progesterone receptors, were transfected with the h*ELF5* promoter-luciferase reporter construct and treated with 10n M 17β-estradiol for up to 48 hours (Figure 5.9).

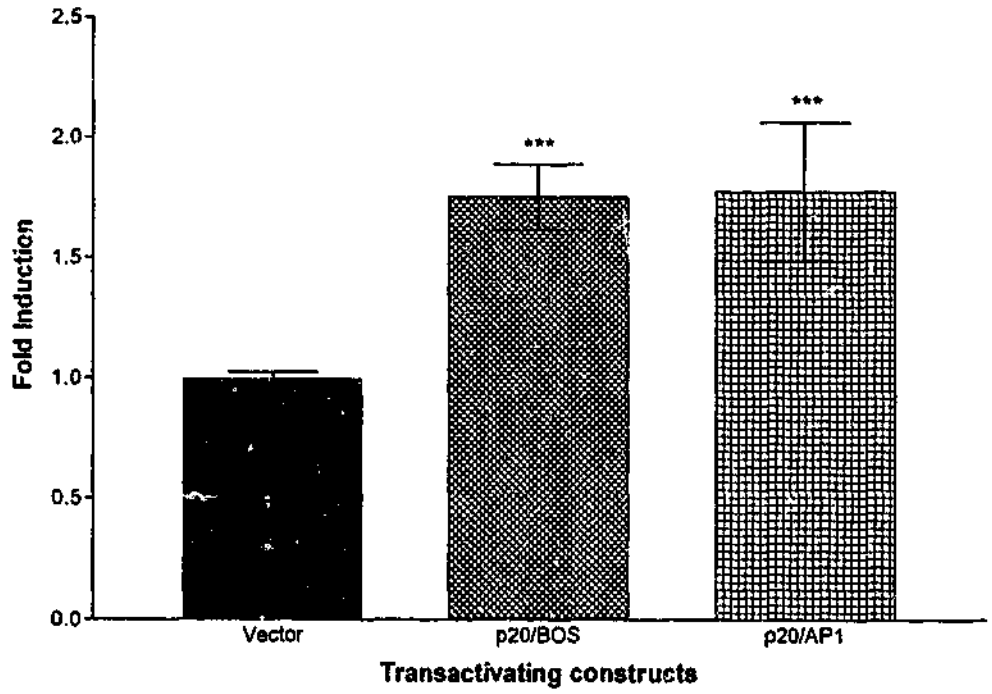


Figure 5.4. AP-1 does not activate the *BRCA1*$^{\alpha}$ promoter synergistically or additively with ELF5 p20. Activity of the promoter construct when co-transfected with empty pEF-BOS vector has been arbitrarily set as 1. n=9. *** p>0.001.

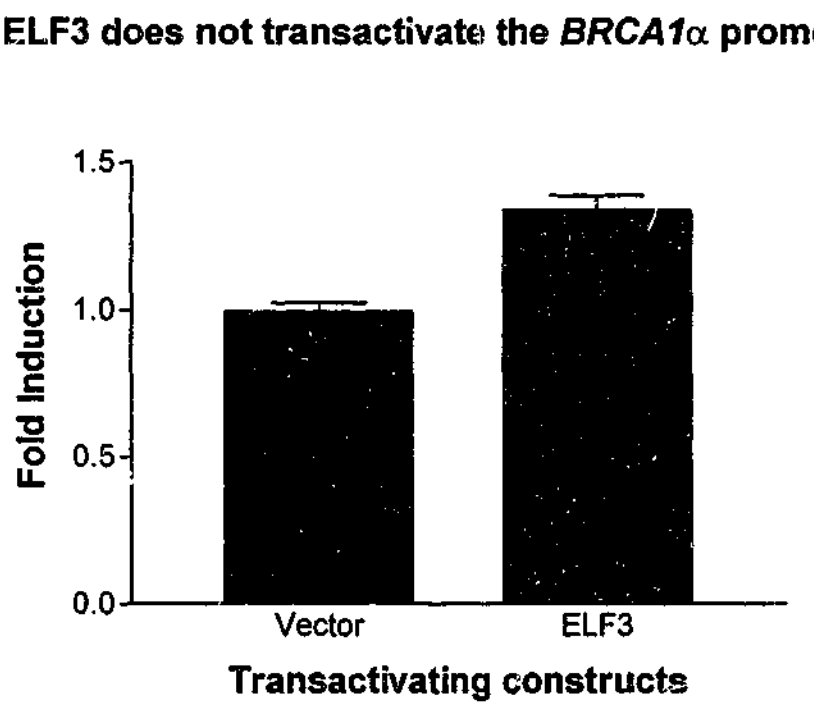


Figure 5.5. ELF3 does not transactivate the *BRCA1*$^{\alpha}$ promoter. Despite the close relationship of *ELF3* to *ELF5*, it does not transactivate *BRCA1*$^{\alpha}$ through any of the EBS in the promoter. Activity of the promoter construct when co-transfected with empty pEF-BOS vector has been arbitrarily set as 1. n=9.

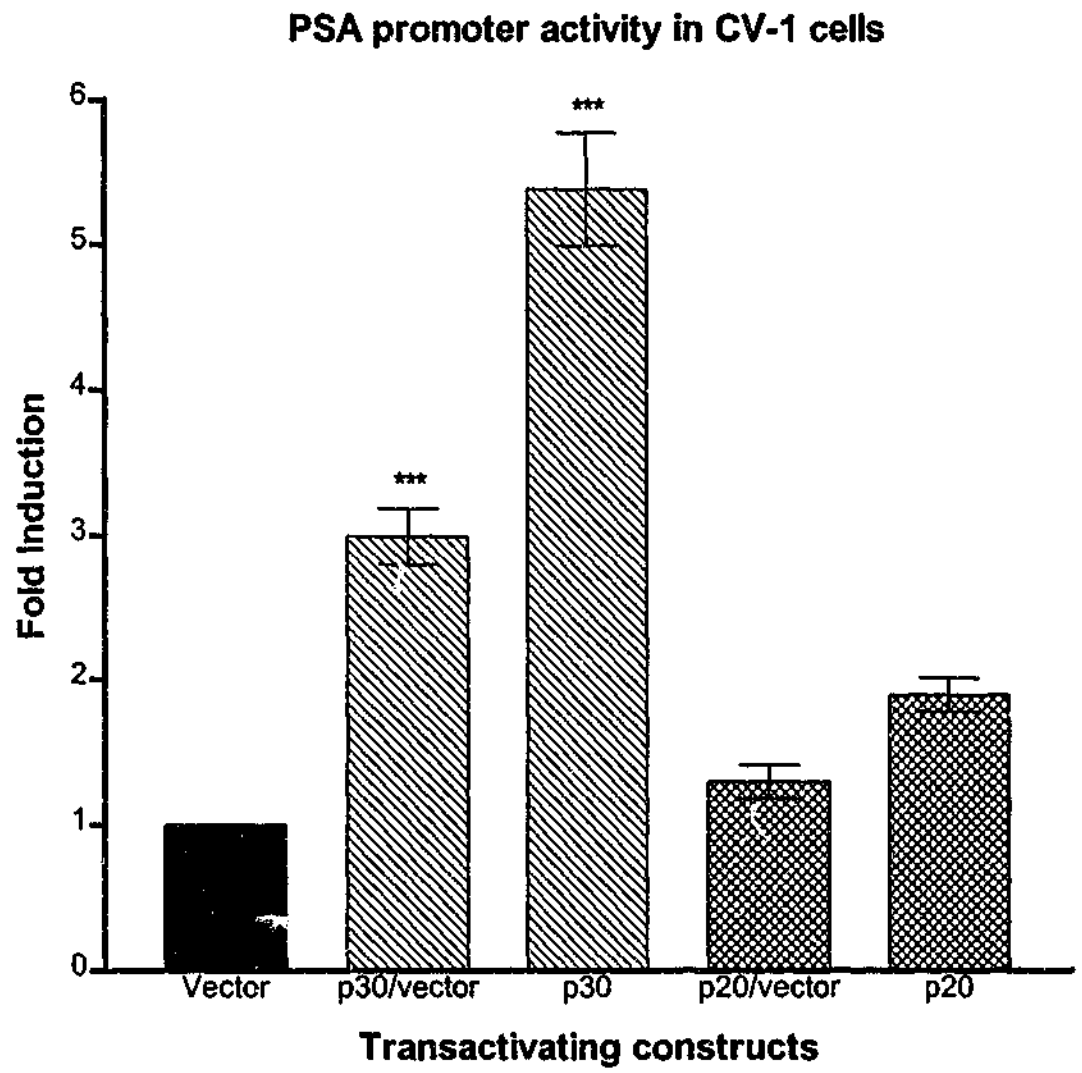


Figure 5.6. p30 transactivates the PSA promoter in CV-1 cells in a dose-dependent manner. Doubling the amount of p30 construct transfected from a 1:1 empty vector:p30 construct mixture (p30/vector) to p30 construct only (p30) doubles the PSA promoter activity. p20 has a negligible effect on the promoter activity. Activity of the promoter construct when co-transfected with empty pEF-BOS vector has been arbitrarily set as 1. n=9. ** p>0.01; *p>0.05.

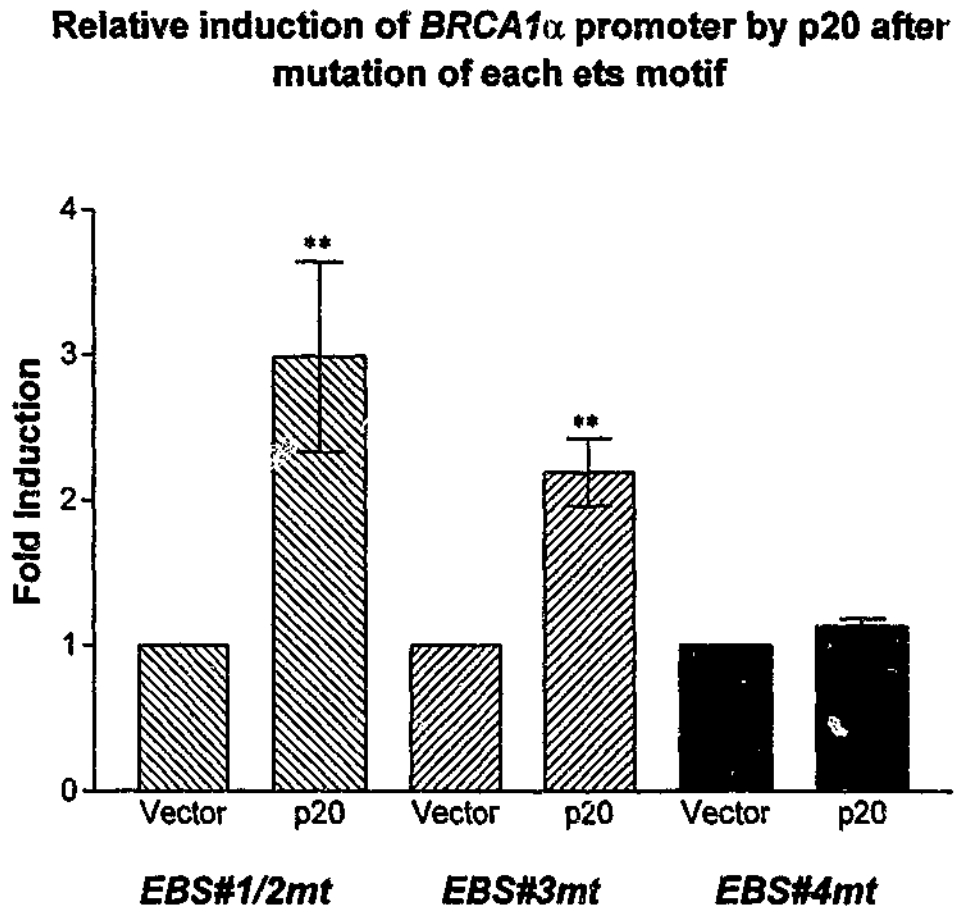


Figure 5.7. Site-directed mutagenesis of each ets-binding motif in the *BRCA1*$^{\alpha}$ promoter demonstrates that p20 acts primarily through the ets#4 site. Transactivation by p20 still occurs when the other two sites are mutated, but is ablated when the ets#4 site is mutated. Activity of the promoter construct when co-transfected with empty pEF-BOS vector has been arbitrarily set as 1. $n \geq 9$. ** $p > 0.01$.

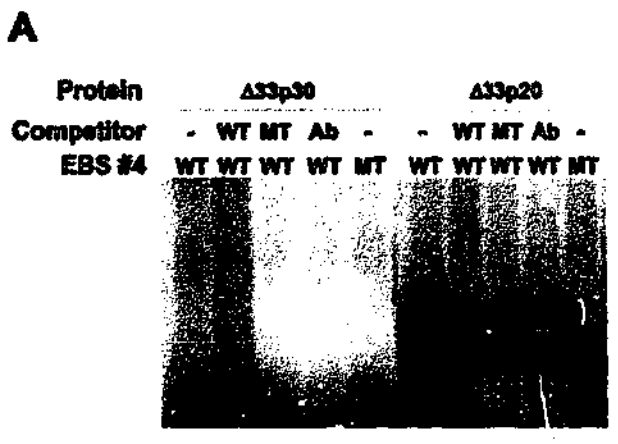


Figure 5.8. EMSA with ELF5 protein isoforms interacting with EBS derived from the BRCA1α promoter (A, B) and with the E74 consensus EBS (C). Δ33 p20 interacts with the BRCA1 ets #4 site (A), with decreased band intensity when competed off with cold competitor oligonucleotide (WT) though not with an antibody directed against the ets domain (Ab). It does not interact with the mutated site, or with the ets #3 site (B). Δ33 p30 does not interact with either *BRCA1α*-derived promoter sequence (A, B), but both proteins bind the consensus E74 oligonucleotide (C), and compete off with wild-type but not mutant competitor oligonucleotide, and with the antibody directed against the *ets* domain.

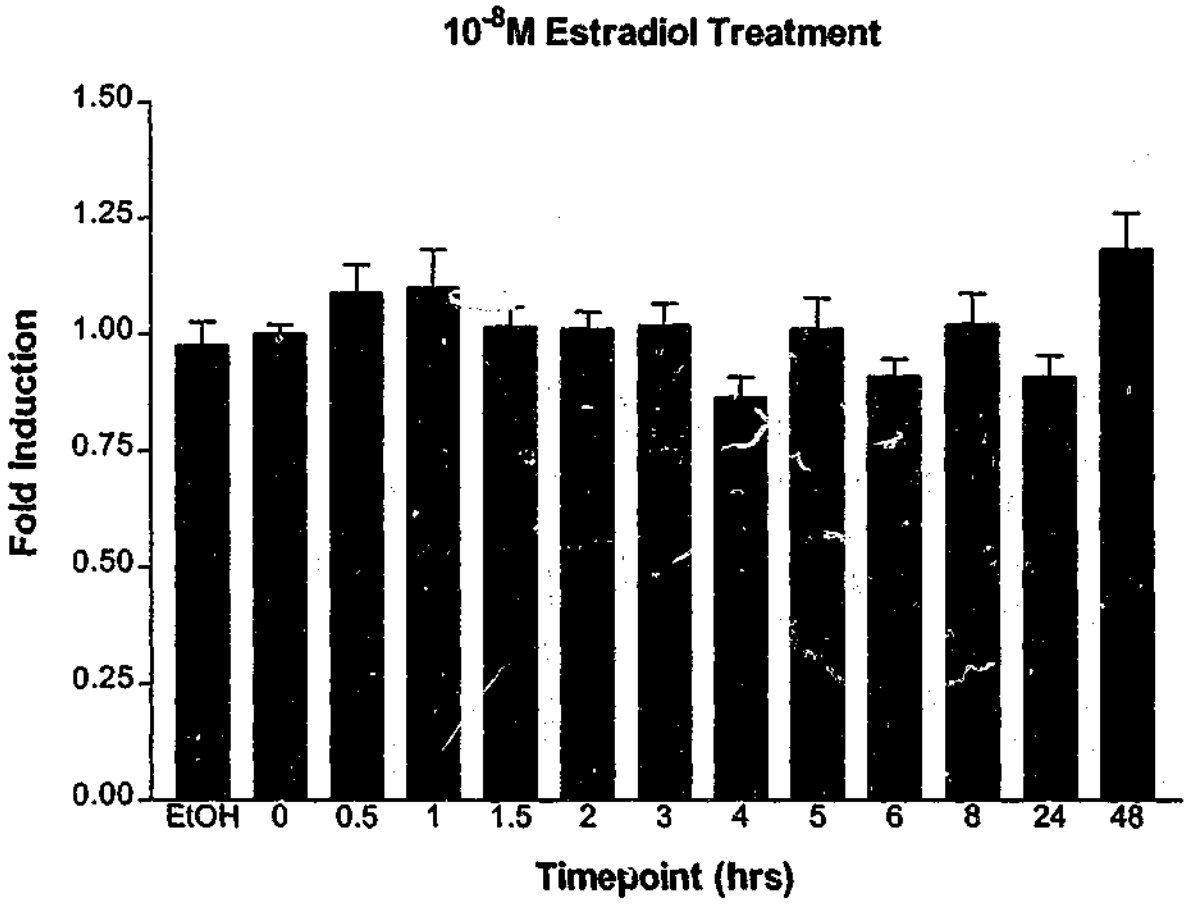


Figure 5.9. The *ELF5* promoter is not estrogen responsive in T47D cells. No significant increase in luciferase activity was noted at any timepoint following treatment with 10 nM 17β-estradiol. n=9. Column 1 (EtOH) represents cells treated with ethanol alone, the solvent for 17β-estradiol.

5.4 Discussion

5.4.1 Regulation of *ets* factors by alternative transcript splicing

The proposition that *ELF5* activity is regulated by alternative splice isoforms arises from a strong historical tradition among *ets* factors. The closely related *ELF* and *ESE* subfamily genes including *NERF/ELF2* (nerf-1a, 1b and 2) (Oettgen et al., 1996; Wilkinson et al., 1997), *ESE-3* (A and B) (Kas et al., 2000) and *Elf3* (Jobling et al., 2002; Tymms et al., 1997) all possess at least two transcripts translated into different protein products. Although all three NERF isoforms bind DNA with similar affinity, NERF-2 transactivates the lymphocytic *lyn* and *blk* promoters while the other two isoforms do not. ESE3b transactivates *C-MET* through two sites in its promoter but ESE-3a has negligible effect on the activity of this promoter. Alternative functions for the two *ELF3* isoforms have not been investigated. Ets-1 produces two isoforms, the full-length p51 and a truncated form, p42, which have distinctly different roles as transcription factors (Baillat et al., 2002). p51 binds a palindromic double *ets* sequence in the *stromelysin-1* promoter, facilitating the formation of an ETS-1:DNA:ETS-1 complex and resulting in transactivation of stromelysin1. p42 lacks exon 7, encoding a domain required for ERK2-dependent phosphorylation (Koizumi et al., 1990) and was therefore inhibited from DNA-binding activity. GABP α and β subunits both possess at least three transcriptional isoforms, produced by exon splicing (de la Brousse et al., 1994; Watanabe et al., 1993); D. O'Leary, PhD Thesis, 2003) but the significance of these is unknown. Three protein isoforms are now known to exist for Elf5; the p20 and p30 isoforms described here and elsewhere (Oettgen et al., 1999b; Zhou et al., 1998) and a longer isoform which uses an alternative exon 1 (Exon 1A), adding ten amino

acid residues to the N-terminal of the protein (ESE-2a) (Oettgen et al., 1999b). These isoforms may provide a type of internal regulation, where the proteins compete for binding on EBS in target promoters.

Transcriptional isoforms of *ets* factors may be expressed tissue specifically, and due to their alternative sequences interact with different protein partners, further increasing the spectrum of regulatory mechanisms employed by these transcription factors. Indeed, the *ELF5* isoform transcribed from exon 1A is expressed mainly in the kidney, while the p30 isoform is predominant in other organs (Oettgen et al., 1999b). We have not yet studied the tissue distribution of the p20 isoform due to the difficulty in obtaining the relevant human tissues, but this should provide data pertinent to the results of this study. To date, we have detected p30 in four (T47D, ZR-75-B, SKBR3 and BT474), and p20 in one (MDA-MB-453), of the five *ELF5*-positive breast cancer cell lines that have been identified (E. Lapinskas, Honours Thesis, 1999). In any one cell line, only one isoform was detected.

We presumed initially that the lack of the pointed domain sequence indicated that p20 would act as a dominant-negative competitor for p30 binding. Clearly this is not the case, but the protein activity depends on the promoter and possibly the cellular context, as shown by the transactivation of the *PSA* promoter by p30 but not p20. While the pointed domain acts as an interface for protein partners, it is not the only interaction domain recorded for *ets* proteins. Different family members possess unique protein interaction domains, but protein interactions involving the *ets* domain have also been recorded (Eisbacher et al., 2003; Fitzsimmons et al., 2001). As *ELF5* does not seem to possess an independent transactivation domain (TAD), it must interact with a protein

that provides this capability. In the context of the *BRCA1α* promoter, p20 and p30 must interact with different protein partners that affect their ability to bind and then to activate the promoter. We have studies in progress to identify proteins that interact with these two isoforms and hopefully provide more information as to how this transcription factor acts on target promoters.

5.4.2 Alternative functions for *ELF5* transcriptional isoforms

One possible explanation for the inability of p30 to transactivate the *BRCA1α* promoter is that it is not a functional transcription factor. We addressed this point by demonstrating that p30 is active in the context of the *PSA* promoter, while p20 is not, once again indicating that it is collaboration between protein partners that determine the outcome on the promoter.

As p20 interacts with an EBS linked to an AP-1 site, we expected that these proteins would interact to potentiate transcription of the promoter, particularly as *ets* proteins commonly interact with AP-1 (Li et al., 2000; Reddy et al., 2003; Ruan et al., 1999). However, either p20 cannot interact with AP-1 or the AP-1 site is not functional. This does not preclude ELF5 interactions with proteins on other parts of the promoter, but we have not yet isolated a candidate interaction.

It is also worthwhile noting that though ELF5 transactivates the bi-directional *BRCA1α/NBR2* promoter in both directions, ELF5 is an epithelial-specific protein with a restricted expression pattern. Therefore, it will only act on the *BRCA1* promoter in tissues where their expression pattern overlaps, such as the breast and kidney epithelium, but will affect transcription of the ubiquitously expressed NBR2 in all

ELF5-expressing tissues. Two isoforms of *BRCA1* are transcribed from the human gene. The *BRCA1α* isoform, studied here, is the major transcript in mammary epithelium. This is particularly interesting as *BRCA1* and *ELF5* transcripts are co-upregulated in the pregnant and lactating mammary gland (Lane et al., 2000; Zhou et al., 1998), and BRCA1 co-operates with BRCA2 to bind STAT5a and suppress its activity on the β-casein promoter (Vidarsson et al., 2002). STAT5a is also crucial for mammary epithelial differentiation and is part of the prolactin receptor (PRLR) signalling pathway, in which ELF5 has also been implicated (Appendix VII).

5.4.3 *ELF5* proteins must be post-translationally modified to be activated

The inability of p20 and p30 ELF5 proteins to bind DNA *in vitro* indicates that the proteins are post-translationally modified within the sequence lying N-terminal to the pointed domain to become activated. Oettgen et al. (1999b) used a similar N-terminal truncated ELF5 protein to ours for EMSA as, unlike ELF3 and ESE-3, full-length Elf5 does not function in this assay (pers. commun.). Many *ets* proteins are the targets of signalling pathways mediated by protein kinases, including ELF5's most similar relative ESE-3, a MAPK target (Tugores et al., 2001). Phosphorylation or dephosphorylation is the most likely explanation for the relief of auto-inhibition of ELF5 interaction with DNA, but this hypothesis has yet to be validated.

5.4.4 *ELF5* regulation of *BRCA1* is not directly regulated by estrogen

BRCA1 transcription is co-ordinately regulated by several factors, including estrogen signalling. Xu et al. (1995) and others propose that *BRCA1α* transcription is regulated by other ER-induced genes, as the translation-blocking reagent, cyclohexamide, ablates

ligand-induced upregulation of *BRCA1* (Marks et al., 1997). We therefore examined ELF5 as a possible ER-induced regulator of *BRCA1α*, but found that the *ELF5* promoter is, surprisingly, not responsive to estrogen, indicating that we must look elsewhere to find such intermediary proteins. Alternatively, ELF5 may still be part of the cascade but be regulated by estrogen-induced genes rather than by direct hormone-receptor complex binding to DNA

5.4.5 Conclusions

We have shown that two distinct isoforms of ELF5 are functional transcription factors that probably depend on protein interactions on DNA to provide their transactivational function. The truncated p20 isoform transactivates the BRCA1α isoform, a protein that has a crucial role in the differentiation of mammary gland epithelial structures, by direct binding to one of the EBS in its promoter, while p30 transactivates PSA, a protein upregulated with malignant transformation of the prostatic epithelium. ELF5 protein appears to require post-translational modification before it is able to interact specifically with DNA, but it is not the missing link between ER signalling and BRCA1α induction.

Chapter 6. *ELF5* is aberrantly expressed in breast, kidney and prostate carcinomas and functions as a tumour suppressor in kidney carcinoma cells

6.1 Introduction

6.1.1 The *ets* oncogene family

Alteration in the expression or activation of *ets* transcription factors is historically associated with tumourigenesis. The first *ets* gene discovered, *ets-1*, was initially characterised as a viral oncoprotein in the E26 avian retroviral genome. The viral sequence was derived from the cellular *Ets-1* gene in the chicken (Nunn et al., 1983). The viral sequence is a truncated version of the cellular *ets* transcript and contains multiple, functionally significant, mutations in both the 5′ and 3′ ends of the coding sequence (Gegonne et al., 1987; Leprince et al., 1983; Watson et al., 1988). The 3′ end of the protein acts as an autoinhibitor of DNA binding in the cellular but not the viral *ets* counterpart (Lim et al., 1992). Therefore degenerate changes in the viral compared to the cellular *Ets-1* sequence result in loss of auto-inhibition and dysregulation of *Ets-1* target genes in the virus-infected cell, leading to oncogenesis.

Subsequent to the discovery of *ets* as an avian viral oncoprotein, the human *ets* homologues, ETS-1 and ETS-2, were found to undergo non-random chromosomal translocations in acute myeloid leukaemia with associated changes in gene expression

(Caubet et al., 1986; Diaz et al., 1986; Le Beau et al., 1986; Papas et al., 1986; Sacchi et al., 1986; Watson et al., 1986b). The characterisation of a plethora of *ets* paralogues in the human genome followed, each of these accompanied by an involvement with cancer. Most of the *ets* genes are expressed exclusively in haemopoietic cells and are hence important in subtypes of leukaemia and lymphoma. The isolation of the epithelial-specific subset of *ets* transcription factors, comprising the closely related cluster of *ELF3*, *ELF5*, *ESE-3* and the divergent prostate-specific *PSE/PDEF*, opened a new door in *ets* cancer biology. While not yet well studied because of their relatively recent advent, these genes may be of tremendous importance considering the prevalence of epithelial-derived cancer among the human population.

6.1.2 The epithelial *ets* factors in carcinoma

The discovery of *ELF3* as an *ets* factor expressed in a wide range of epithelial structures in various organs was paralleled by genomic DNA and mRNA expression studies in epithelial cancer. Chang et al. (1997) noted the over-expression of *ELF3* mRNA in the early stages of breast tumourigenesis and the associated amplification of the chromosome 1q32.1 locus where *ELF3* is situated. Likewise, Tymms et al. (1997) studied *ELF3* expression in lung cancer cell lines and found increased expression in lung carcinoma compared with normal tissue. A positive feedback loop between the proto-oncogene ErbB2 and *ELF3* has been postulated in the subset of breast cancers that over-express *ELF3*. ErbB2 signalling leads to increased *ELF3* promoter activity and protein levels, and ELF3 subsequently transactivates the *ErbB2* promoter (Neve et al., 2002). ELF3 expression is also required for the survival of non-transformed and

transformed breast cell lines MCF-12A and T47D (Eckel et al., 2003). These data indicate that *ELF3* may be oncogenic when dysregulated in breast epithelium.

Further work from Chang et al. (2000) established the cytokine receptor TGFβRII as a positively regulated transcriptional target of *ELF3,* providing a clue to the role of *ELF3* in breast cancer. A model was proposed whereby decreased TGFβRII expression in cancer cells is permitted by down-regulated *ELF3* expression, resulting in resistance to the growth-inhibiting effects of TGFβ signaling (Chang, 2000). Similar data were obtained in a gastric cancer cell line system (Park et al., 2001). *ELF3* has been proposed as a retinoic acid (RA) response gene, where RA is an important agent in cancer treatment and prevention (Ma et al., 2003b). This model, however, proposes that *ELF3* is not an oncogene, but a tumour suppressor, which is not concordant with the over-expression of *ELF3* in cancer. However, the expression analysis did not account for possible mutations in the transcript that could lead to a non-functional protein product, a hypothesis that could reconcile the two viewpoints. The data on the role of *ELF3* in cancer is conflicting and possibly contradictory. While studies continue that will contribute to this work, it seems likely that though the function of *ELF3* in carcinoma has not been clearly delineated, a role for it does exist.

Murine *Ese-3* was first isolated from pituitary somatotroph tumours (Bochert et al., 1998). The role of the gene in cancer has not been well-studied, but in development it is believed to function in glandular epithelial differentiation and branching morphogenesis via promoter regulation of *c-met* (Kas et al., 2000), and to repress transcription of target genes following MAPK phosphorylation (Tugores et al., 2001). Dysregulation of these functions has the potential to lead to dysmorphic breast epithelial structures and to

inappropriate de-repression of target genes, both of which may be conducive to cancer initiation and/or progression.

Initially believed to be prostate specific (Oettgen et al., 1997b; Yamada et al., 2000), the phylogenetically divergent *ets* protein, PSE, is found in epithelial-rich organs including breast, prostate and colon (Feldman et al., 2003a). It is aberrantly regulated in transformed prostate glandular structures compared with non-cancerous prostate. Nozawa *et al.* (2000) noted that while *PSE* mRNA is expressed in normal prostate glandular epithelium as well as the prostate carcinoma cell lines PC3 and LNCaP, the protein is only detectable in the normal prostate tissue samples, not in the cell lines, implying a specific translational control mechanism for this mRNA. PSE transcriptionally activates prostate-specific genes including the prostate cancer marker, *PSA*, which it activates in an androgen-independent manner. PSE also directly interacts with the DNA-binding domain of the androgen receptor (AR) to synergistically enhance androgen-mediated *PSA* transactivation (Oettgen et al., 2000). As discussed in Chapter 1, over-expression of the *PSE* transcript is a novel marker for metastatic breast cancer in lymph nodes, and is a breast cancer circulating antigen (Ghadersohi and Sood, 2001; Mitas et al., 2002). PSE protein levels, however, are reduced in invasive breast cancer and re-expression of the protein in breast cancer cells resulted in cell cycle arrest, inhibiting cell growth, migration and invasion (Feldman et al., 2003a). These data indicate that PSE is a tumour suppressor gene, and that the loss of PSE protein in breast epithelium alters the expression of a spectrum of genes that control breast cancer progression and invasion (Feldman et al., 2003a).

Less is known about a potential role for *ELF5* in cancer progression, although the gene was isolated at approximately the same time as *ELF3* and prior to the discovery of *ESE-3* and *PSE*. *ELF5* mRNA was not expressed in a panel of carcinoma cell lines derived from breast, kidney, lung and prostate; organs in which it is normally expressed (Zhou et al., 1998). The only two cell lines that retain expression are the hormone-responsive, non-aggressive lines T47D (breast carcinoma) and LNCaP (prostate carcinoma). This may indicate that loss of *ELF5* mRNA is associated with conversion to a more aggressive, invasive or hormone-independent phenotype. Evidence for genomic alterations of *ELF5* alleles as evaluated by Southern blotting was found in four of the nine lung carcinoma cell lines tested, and amplification of the *ELF5* locus was found in the breast carcinoma cell line, T47D (Zhou et al., 1998). QRT-PCR analysis of *ELF5* message in primary breast cancers showed >2-fold under-expression compared to normal breast in 95% of samples (Ma et al., 2003a), which is consistent with the cell line data.

Hypermethylation and LOH at *Homo. sapiens* chromosome (HSA) 11p13, where *ELF5* is localised, is a common phenomenon in a variety of epithelial-derived solid tumours (Baffa et al., 1996; de Bustros et al., 1988; Gudmundsson et al., 1995; Shipman et al., 1998; Shipman et al., 1993; Winqvist et al., 1995), and the chromosomal region harbours defined and undefined tumour suppressor elements (Goldberg et al., 2000; Harris et al., 1991; Lee and Haber, 2001).

Elf5 heterozygote mice show defects in pregnancy-associated mammary gland development; in the formation of mature lobuloalveolar structures. Whether the defect affects the proliferation or the differentiation of the epithelial cells is not known

(Appendix VII). This indicates that *Elf5* is necessary for normal mammary epithelial cell development and that, like other *ets* factors, dysregulation may result in the inappropriate expression of target genes and altered cell morphogenesis.

These data suggest that *ELF5* may be a tumour suppressor gene. Tumour suppressors are defined by the following characteristics, although not every tumour suppressor will demonstrate all of the qualities. Tumour suppressors undergo loss of function in cancer, by LOH, decreased transcription or translation, or mutation in the protein product. They can be mutated in inherited cancer syndromes and may be able to inhibit the growth, tumourigenesis or metastasis of transformed cells *in vitro* or *in vivo*.

We hypothesise that *ELF5* is a tumour suppressor gene. Like other tumour suppressor genes, the mRNA and protein expression of *ELF5* may be decreased in epithelial-derived cancer, particularly of the breast, kidney and prostate, due to cancer-associated transcriptional deficits. As is the case for other genes at 11p13, defective transcription of *ELF5* may be due to LOH or possibly to promoter hypermethylation; this criterion for tumour suppressor activity will be tested in our study using genomic DNA derived from carcinomas. *ELF5* may suppress the tumourigenicity of transformed breast and kidney epithelial cells following re-introduction into these cell lines. Suppression of the ability to grow as anchorage-independent colonies in culture will be assessed by the *in vitro* soft agar assay, and tumour-forming capacity assessed by the *in vivo* SCID mouse assay.

6.2 Methods

6.2.1 Isolation and analysis of an *ELF5*-linked microsatellite marker

Following the isolation of a functional *ELF5* promoter region, the DNA sequence was analysed for repetitive sequences using the RepeatMasker program (Baylor College of Medicine Search Launcher, http://searchlauncher.bcm.tmc.edu). Following identification of repetitive 2-4 nucleotide elements, oligonucleotides were designed flanking the microsatellite repeats. A $GAAA_{\sim 50}$ repeat was analysed for polymorphism in 100 chromosomes from normal blood samples (Chapter 2) obtained from the Victorian Clinical Genetics Service (VCGS), using oligonucleotides 37 and 38 (Appendix I). Briefly, the oligonucleotide 37 was labelled with [γ-^{33}P]-dATP (10 mCi/ml; Amersham-Pharmacia) as described in Chapter 2. Genomic DNA (100 ng) was used as a template for PCR, where labelled and unlabelled oligonucleotide 37 were used in a 1:5 ratio to a final concentration of 0.75 μM. Oligonucleotide 38 was also added to a final concentration of 0.75 μM. *Pfx* polymerase (Invitrogen) was used to amplify the microsatellite marker according to the manufacturer's instructions. Following 35 cycles of PCR, 0.2 volumes of formamide DNA loading dye (95 % (v/v) deionized formamide; 10 mM EDTA (pH 8.0); 0.1 % (w/v) bromophenol blue; 0.1 % (w/v) xylene cyanol) was added to each PCR reaction and boiled for 5 min. Five μl of each PCR was electrophoresed through a 6 % (w/v) urea/polyacrylamide gel (6 % (w/v) poly-acrylamide; 0.5 X TBE; 7 M urea) at 2500 V and 50°C for 3 hours using a Bio-Rad Sequi-Gen I apparatus. The gel was dried onto Whatman (3MM chromatography) paper and exposed to Biomax MR (Kodak) film for 1 week at –80°C.

Genomic DNA (100 ng) from 20 breast tumours and patient-matched peripheral blood lymphocytes (PBLs) was obtained from the Peter MacCallum Tissue Bank (Victoria, Australia). PCR and urea-PAGE analysis was performed as above. For informative individuals, allele intensity was compared between tumour and PBL samples to determine whether LOH occurred at the locus, where a decreased or absent allele in the tumour compared with normal DNA was indicative of LOH.

6.2.2 Production of the pBI-EGFP-*ELF5* plasmid

The pBI-EGFP vector was obtained from Clontech. *Nco*I/*Bst*XI-digested *ELF5* p30 and p20 cDNAs were blunt-ended and cloned in the forward orientation into the *Pvu*II site of the vector.

6.2.3 Production of MCF7 breast and 786-O renal carcinoma cell lines expressing ELF5

MCF7 cells were maintained in culture as described in Chapter 4. 786-O cells were also maintained in RPMI 1640 medium with 10 % (v/v) FCS. All transfections were performed using FuGene 6 transfection reagent (Roche). MCF7 cells were initially co-transfected with a 5:1 molar ratio of the pRev-TRE and pTK-hyg plasmids (Clontech) encoding the rtTA receptor and hygromycin antibiotic resistance respectively, and clonal populations were selected using 100 μg/ml hygromycin B. Plasmids had been linearised by restriction endonuclease digestion with *Sca*I and *Hin*dIII respectively and gel purified prior to transfection. Once expression of the receptor was confirmed by RT-PCR with Tet-On specific primers (oligonucleotides 43 and 44, Appendix I), a second round of transfection was performed in this sub-line to introduce the *Nhe*I-

linearised pBI-EGFP-*ELF5* vector. Double-transfected clones were selected with 500 μg/ml G418 and 100 μg/ml hygromycin B.

Cells (786-O) were co-transfected with *Nco*I-digested *ELF5* p20 or p30 in the pEF-BOS plasmid (Mizushima and Nagata, 1990) and *Eco*RI-digested puromycin-resistance plasmid (Ramirez-Solis et al., 1995). Clonal lines were selected with 3 μg/ml puromycin (ICN Biochemicals).

6.2.4 Testing expression of ELF5 and EGFP in transfected cell lines

In MCF7 transfected cells, EGFP expression was detected before and after doxycycline induction (2 μg/ml; 48 hours) by fluorescence microscopy using a green fluorescent protein (GFP) (488 nm) filter. Nuclei were counterstained with 1 ng/ml 4'-6-diamidino-2-phenylindole (DAPI) in PBS (Sigma) for 10 min and visualised under UV light microscopy. Enhanced green fluorescent protein (EGFP) expression profiles were compared between doxycycline-induced and non-induced clones by fluorescence-activated cell sorting (FACS) analysis using $1x10^6$ cells resuspended in PBS. Expression of *ELF5* and *EGFP* mRNAs was confirmed by RT-PCR and the presence of *GAPDH* mRNA was used as a positive control for reverse transcribed cDNA. RNA extractions and reverse transcription were performed as described in Chapter 2. *ELF5* oligonucleotides 49 and 50 (Appendix I) distinguished between p20 and p30 isoforms by producing different size amplimers of ~60 bp (p20) and ~360 bp (p30). *EGFP* was detected using oligonucleotides 47 and 48. *GAPDH* was detected using oligonucleotides 41 and 42.

6.2.5 Monolayer growth rate of transfected cells

Growth rates of mock transfected and *ELF5* transfected cell lines were compared by colourimetric assay using the mitochondrial dye 3-(4,5- dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were seeded in 96 well clear plates (BD Labware) at $1x10^3$ and $2.5x10^3$ cells per well in triplicate. Two different cell concentrations were used to obtain the optimal growth curve for each cell line. At day 0 (4 hours post-seeding), and days 1-4, 50 µl 2 mg/ml MTT in PBS was added to each well and incubated for 3 hours in cell culture conditions. Media and MTT was removed and cells lysed with 150 µl dimethylsulfoxide (DMSO). Absorbance readings at 420 nm were analysed on the FluoStar Optima (BMG Laboratory Technologies). Assays were repeated three times, independently.

6.2.6 Growth of transfected cells in anchorage-independent conditions

Growth of *ELF5* transfected clones was compared with mock transfected clones when resuspended in soft agarose; that is, without attachment to the plastic plate. Two ml of 0.7 % (w/v) agarose in culture medium was plated in 6 well plates (BD Labware). $1x10^4$ cells were resuspended in 1 ml of 0.35 % (w/v) agarose in culture medium and layered upon the 0.7 % (w/v) bottom agarose support. Wells were overlayed with 2 ml culture medium and incubated at 37°C/5 % CO_2 for 21 days, with changes of medium 2-3 times per week. Each clone was plated in triplicate, and the assay repeated three times independently. To score the assays, three images of each well were taken and colonies counted as small (cells that have not divided more than twice) or large (cells that have divided more than twice) to account for total number of cells seeded per field of vision, and for the proportion that had expanded into anchorage-independent growing colonies.

6.2.7 Tumourigenic capacity of transfected cells *in vivo*

The capacity of *ELF5*-transfected renal carcinoma cell clones, compared with mock-transfected cells, to form tumours in immunocompromised mice was tested by subcutaneous cell inoculation. $1x10^6$ 786-O derived cells were resuspended in 100 µl Matrigel (BD BioSciences) and injected subcutaneously under the hind leg of 5-6 week old female SCID (Severe Combined Immuno-Deficiency) mice (Animal Research Council, WA, Australia). The tumours were measured twice a week, and the tumour size (mm^3) was evaluated by measurement of three perpendicular diameters with microcalipers and using the formula 4/3 LxWxH, where L is the longest diameter and W is the diameter perpendicular to L, and H is the height of the tumour. When tumours reached an L diameter of 10 mm, the mice were culled and tumours harvested. Half the tumour was snap-frozen for molecular biology analysis (Chapter 2) and half was fixed in formalin and paraffin-embedded for histological and immunohistochemical analysis as described in Chapter 3.

6.2.8 RT-PCR analysis of *ELF5* and *GAPDH* in normal organ RNA

Human organ total RNA was obtained from Stratagene. Five µg total RNA was reverse transcribed as described in Chapter 2. *ELF5* was detected using primers designed in the 3'UTR as described in Ma et al. (2003) (oligonucleotides 51 and 52, Appendix I). PCR products were transferred to Genescreen Plus (NEN) membrane by alkaline transfer and probed with an internal oligonucleotide (oligonucleotide 53) as described in Chapter 2. *GAPDH* control for the presence of reverse transcribed cDNA was performed using primers 41 and 42.

6.2.9 Quantitative RT-PCR analysis of breast carcinoma and renal carcinoma cell lines

Eighteen matched breast tumour RNA and normal breast RNA samples were obtained from the Peter MacCallum Tissue Bank. cDNA was prepared as described in Chapter 2. Twenty breast tumour cDNAs, four with matched normal breast cDNA, were a kind gift from from Jonathan Cebon and Suzanna Svobodova (Ludwig Institute for Cancer Research Oncology Unit, Melbourne). Normal reference breast RNA was obtained from Stratagene. Nineteen renal cell carcinoma cDNAs were also supplied by Jonathan Cebon and Suzanna Svobodova. Normal reference kidney RNA was obtained from Stratagene. QRT-PCR was performed on the LightCycler (Roche) using FastStart DNA Master SYBR Green I (Roche) according to the manufacturer's instructions. *ELF5*-18 and 19 RT-PCR primer sequences are described above. Amplification took place in 3 mM $MgCl_2$, with 10 second (sec) denaturation at 95°C, 5 sec annealing at 55°C and 11 sec extension at 72°C. *Cytokeratin 8* was used as a marker for simple epithelium (oligonucleotides 45 and 46). Amplification was performed as for *ELF5* but with 5 sec annealing at 59°C and 8 sec extension at 72°C. *BRCA1* mRNA levels were measured in breast tumour and normal samples using oligonucleotides 47 and 48. Amplification was performed as for *ELF5* but with a 16 sec extension at 72°C. *ELF5* and *BRCA1* expression was normalised to *Cytokeratin 8* expression levels to compare between samples.

6.2.10 Protein expression of ELF5 in cancer sections

Anti-ELF5 immunohistochemistry on formalin-fixed, paraffin-embedded, 5 µm cancer sections was performed as described in Chapter 3. Breast and renal carcinoma 2 mm cancer biopsy slides were obtained from SuperBioChips Laboratories (Seoul, South

Korea). Prostate carcinoma radical biopsy slides were provided and stained by Qihan Dong (University of Sydney).

6.3 Results

Our hypothesis, based upon preliminary evidence from our laboratory and others, is that *ELF5* is an *ets* transcription factor with tumour suppressor capability. As *ELF5* expression was not detected in a variety of epithelial-derived cancer cell lines (Oettgen et al., 1999b; Zhou et al., 1998) and the genomic locus may have been rearranged in some of these, we searched the locus in a more detailed fashion for LOH of *ELF5* in primary breast cancer DNA rather than cell lines, which are a less reliable indicator of the *in vivo* situation.

6.3.1 LOH analysis for ELF5 in breast cancer

A microsatellite marker, $GAAA_{\sim 50}$, was identified approximately 6 kb 5′ to *ELF5* exon 1. The polymorphism information content (PIC) of the marker was analysed in the general population using 100 normal chromosomes derived from 50 blood samples. Thirteen different length alleles of the microsatellite marker were observed amongst the sample population, with 86% of the samples showing heterozygosity at the marker (Figure 6.1A). Given the large number of alleles identified at this marker, the PIC was 0.86, or equivalent to the percentage of individuals heterozygous for the marker. Eighteen patient cases of breast tumour DNA and blood derived from PBLs were then analysed for heterozygosity at the GAAA microsatellite marker (Figure 6.1B). Of these, 16 cases were informative, whereas two were homozygotes at the marker and

could not be analysed. None of the 16 informative cases showed loss or significant decrease in intensity of an allele in the tumour compared with normal DNA, indicating that the locus is not undergoing LOH in primary breast cancers.

A second possibility that may account for the lack of expression of *ELF5* in epithelial-derived carcinoma cell lines is hypermethylation of the promoter region, which is associated with gene silencing. To investigate the possibility that the *ELF5* promoter was hypermethylated in cancer cell lines, we attempted to optimise the bisulphite sequencing method (Everett and Chambon, 1982; Warnecke et al., 2002) on the *ELF5* promoter. To date, this work has not been successful and the technique requires further optimisation for the DNA sequence of interest before we can determine whether the promotor is aberrantly methylated in cancer cell line or primary tumour DNA compared with normal genomic DNA.

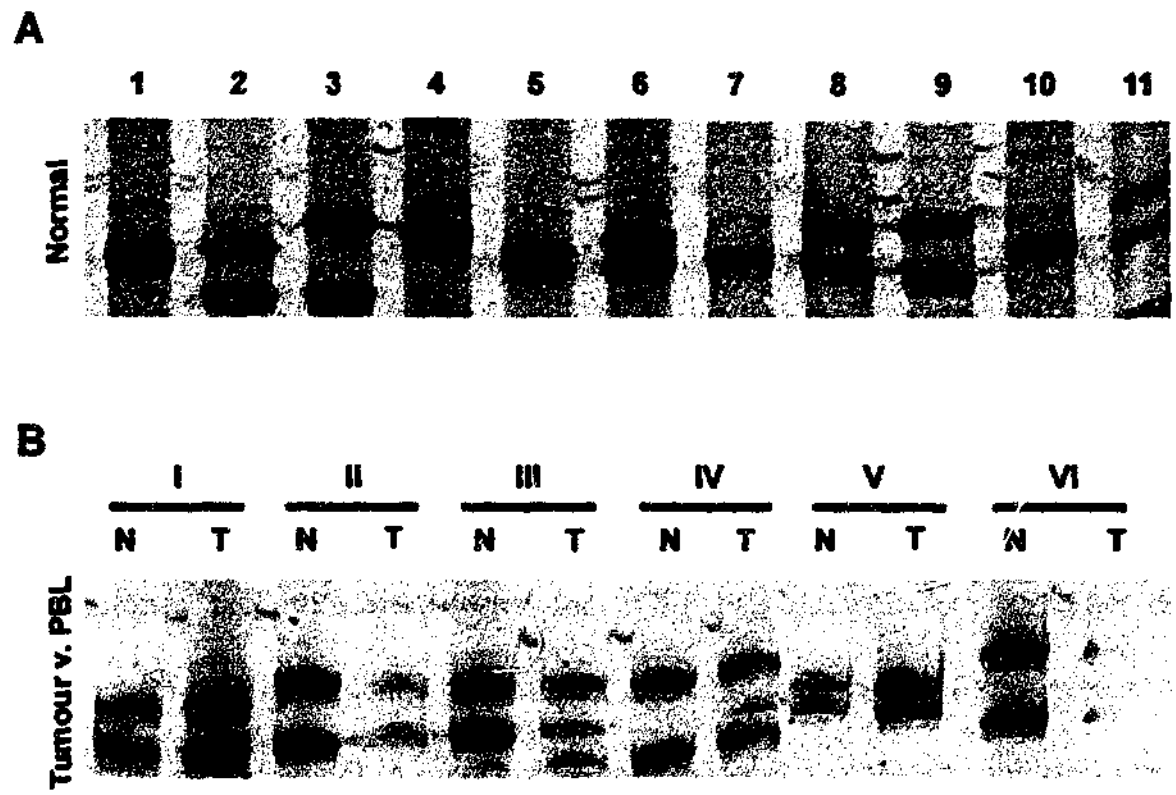


Figure 6.1. A GAAA tetranucleotide repeat microsatellite marker was analysed for heterozygosity by ^{33}P-labelled PCR and urea-PAGE analysis. Eleven individuals of the 50 screened for heterozygosity are shown in (A). Cases 2-3; 5-6; 8-9 and 11 represent heterozyogous for different length alleles of the marker, while cases 1, 4, 7 and 10 are homozygous at the marker. Six representatives are shown of screening for heterozygosity in PBL DNA (N) compared with breast tumour DNA (T) (B). All 6 cases are heterozygous at the marker in their normal DNA but have not lost an allele in the tumour DNA, indicating that the locus is not undergoing LOH in breast cancer.

6.3.2 Effect of re-expressing *ELF5* in breast carcinoma cells and kidney carcinoma cells on the tumourigenic phenotype

Breast ductal carcinoma MCF7 Tet-On cells expressing the intracellular tetracycline receptor rtTA were transfected with *ELF5* p20 and p30-containing pBI-EGFP vectors (Figure 6.2A). These vectors contain the CMV promoter linked to a tetracycline-responsive site. The promoter drives *EGFP* gene expression in one direction and *ELF5* in the other direction. The tetracycline-responsive site is bound by rtTA in concert with tetracycline (tet) or its analogue, doxycycline (dox), thereby inducing *EGFP* and *ELF5* gene expression.

Subcloned cell lines were tested for EGFP expression before and after doxycycline induction by fluorescence microscopy and FACS analysis. Fluorescence microscopy detected EGFP signal in both uninduced (data not shown) and induced clones (Figure 6.2C), indicating that EGFP was being expressed in the absence of doxycycline induction and that the inducible promoter system was leaky. The EGFP expression profile of the cells was then examined by FACS analysis to detail whether doxycycline induction induced the expression of the marker gene and henceforth of *ELF5*. FACS analysis showed higher fluorescence in pBI-EGFP-ELF5 transfected cells than in mock transfectants and that 20-25 % of the cells responded to doxycycline induction by increasing EGFP expression. Therefore, the ELF5 transfectants expressed ELF5 in the uninduced state but responded to doxycycline induction by increasing gene expression. Likewise, ELF5 transfectants showed *EGFP* and *ELF5* mRNA expression in both uninduced and induced states, whereas mock transfectants do not (Figure 6.2B). Renal

carcinoma cells (786-O) were constructed to constitutively express *ELF5* by stable transfection with *ELF5* isoforms under the control of the *EF1α* promoter (Figure 6.3).

Monolayer growth rates of the transfected MCF7 and 786-O cells were compared with those of the *ELF5*-expressing sub-clones by MTT assay (Figure 6.4). The expression of *ELF5* isoforms in MCF7 or 786-O cells had no significant effect on growth rate in a monolayer.

Growth of the cell lines in three-dimensional, anchorage-independent conditions demonstrated a difference in this environment compared with the monolayer, two-dimensional growth. Approximately 90 % of mock transfected MCF7 and 786-O cells formed large colonies. While p20 and p30 *ELF5*-expressing MCF7 cells showed no significant difference in colony formation ability from the mock transfected cells (data not shown), p30-expressing 786-O cells showed a significantly decreased (71 %) proportion of cells that formed large colonies compared to mock transfectants ($p>0.001$) (Figure 6.5A). p20 transfectants demonstrated a 22 % reduced proportion of cells that formed large colonies compared with mock transfectants ($p>0.05$). Qualitatively, colony size also appeared to be decreased in *ELF5* transfected 786-O cells compared with mock transfectants (Figure 6.5B).

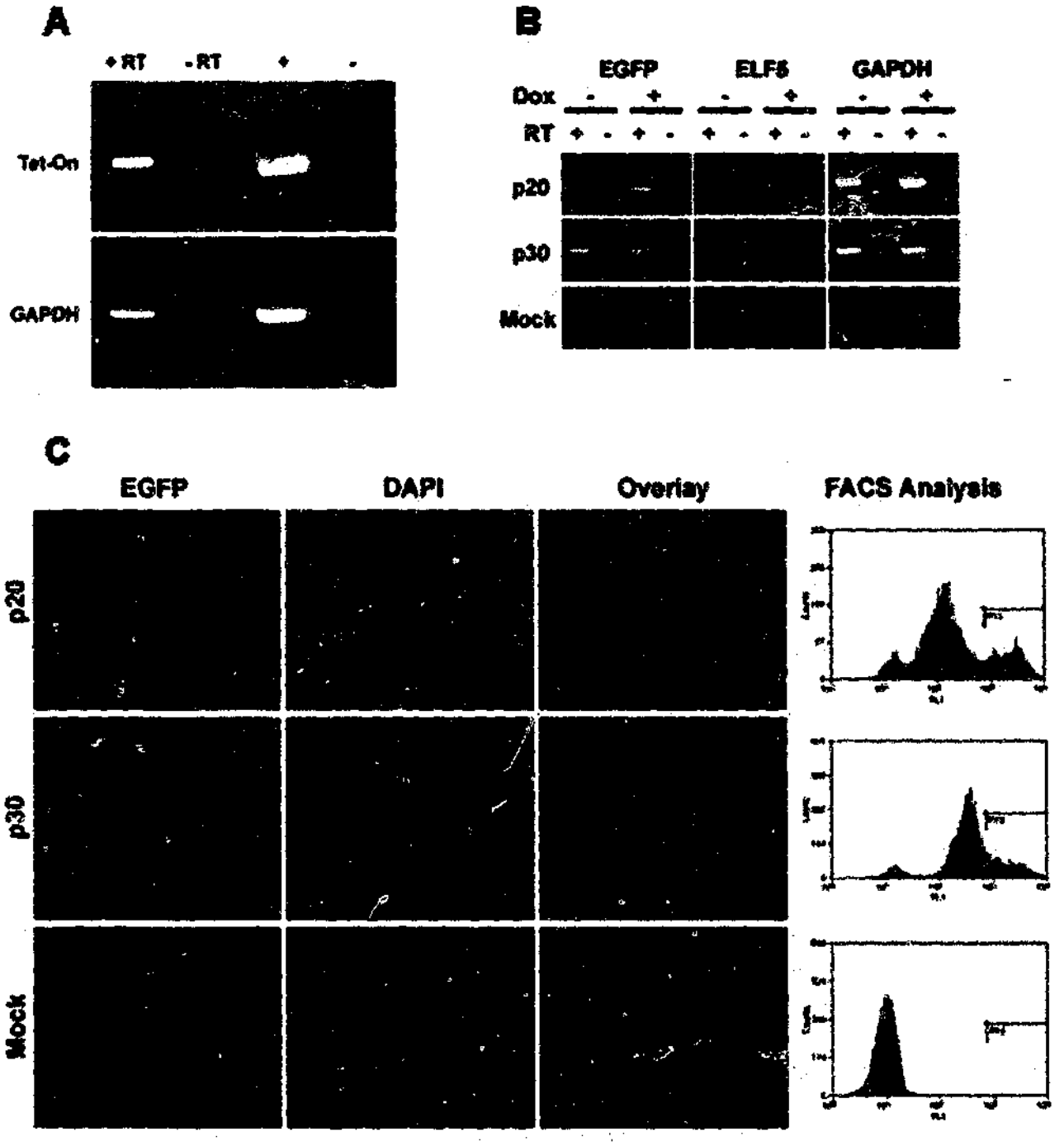


Figure 6.2. Production of MCF7 cells expressing *EGFP* and *ELF5* in an inducible fashion. Data from representative clones is shown. MCF7 cells stably expressing the rt-TA tetracycline/doxycycline internal receptor were produced and verified by RT-PCR (A). Following transfection of the ELF5-pBI-EGFP vector into the Tet-On cell line, sub-clones were screened for *EGFP* and *ELF5* mRNA expression by RT-PCR, prior to and following doxycycline induction (B). EGFP fluorescence was confirmed by microscopy and FACS analysis after doxycycline induction (C). The R13 window shows the proportion of cells with increased fluorescence following dox induction. *GAPDH* was used as a control for the presence of reverse transcribed cDNA. Plasmids containing the relevant DNA were used as positive PCR controls (+) and water as a negative control (-).

As the results from the soft agarose assay indicated that *ELF5* might be inhibiting anchorage-independent growth in renal carcinoma cells, but not in breast carcinoma cells, the 786-O transfectants were inoculated subcutaneously into SCID mice to analyse the tumour-forming capability of the cells. In two independent experiments (a third experiment was rejected as the mock-transfected cells did not form tumours), 90% of mock transfectant-inoculated mice established tumours, while the size of the inoculated sites in 90% of p30-transfectant mice decreased steadily post-inoculation until lumps were no longer detectable. The other 10% of mice maintained tumour growth equivalent to that of the mock-transfectant injected mice. 70% of p20-transfectant inoculated mice developed and maintained tumour growth (Figure 6.6). Analysis of the inoculation sites of mock, p20 and p30 786-O cells showed formation of solid tumours, containing closely packed epithelial-like cells and blood infiltrate, in mock and p20 injected mice. Sparse, small epithelial-like cell clusters were identified in the injection sites of the p30 injected mice, dispersed among the subcutaneous fat (Figure 6.7). Blood vessels and infiltrate were not observed in the p30 as they were in the mock and p20 injection sites. Anti-ELF5 immunohistochemistry showed strong staining around epithelial-like cells in p20 and p30 injection sites, but not in mock transfectant injection sites. While high background staining was observed, due to non-specific antibody adsorption to the subcutaneous fat, cell-associated staining showed a distinct increase in the ELF5-transfected injection sites (Figure 6.7), indicating that the cells forming the tumours in the injection sites are indeed the 786-O transfectants.

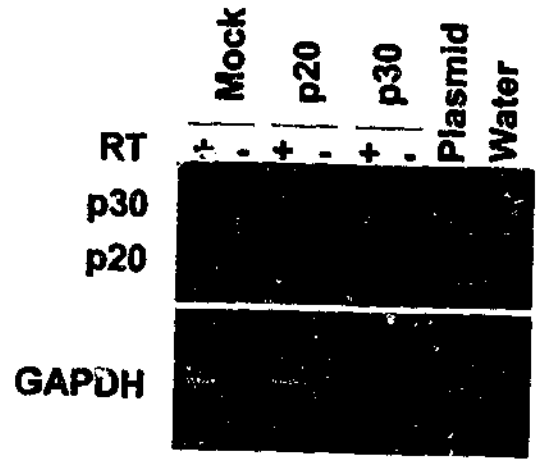
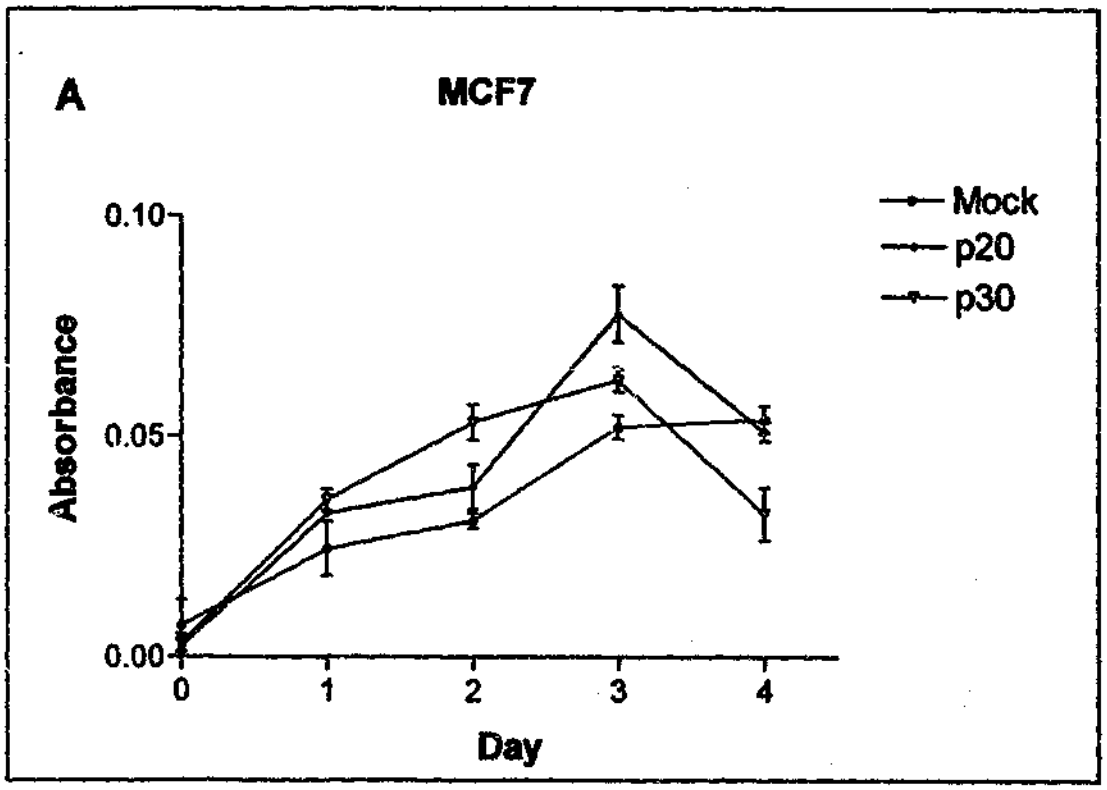


Figure 6.3. RT-PCR characterisation of *ELF5*-transfected 786-O renal carcinoma cells. Mock, *ELF5* p20 and *ELF5* p30 transfected cells were analysed for expression of *ELF5* and *GAPDH* mRNA by RT-PCR. *GAPDH* was used as a control for the presence of reverse transcribed cDNA. Plasmids containing the relevant DNA were used as positive PCR controls (+) and water as a negative control (-).



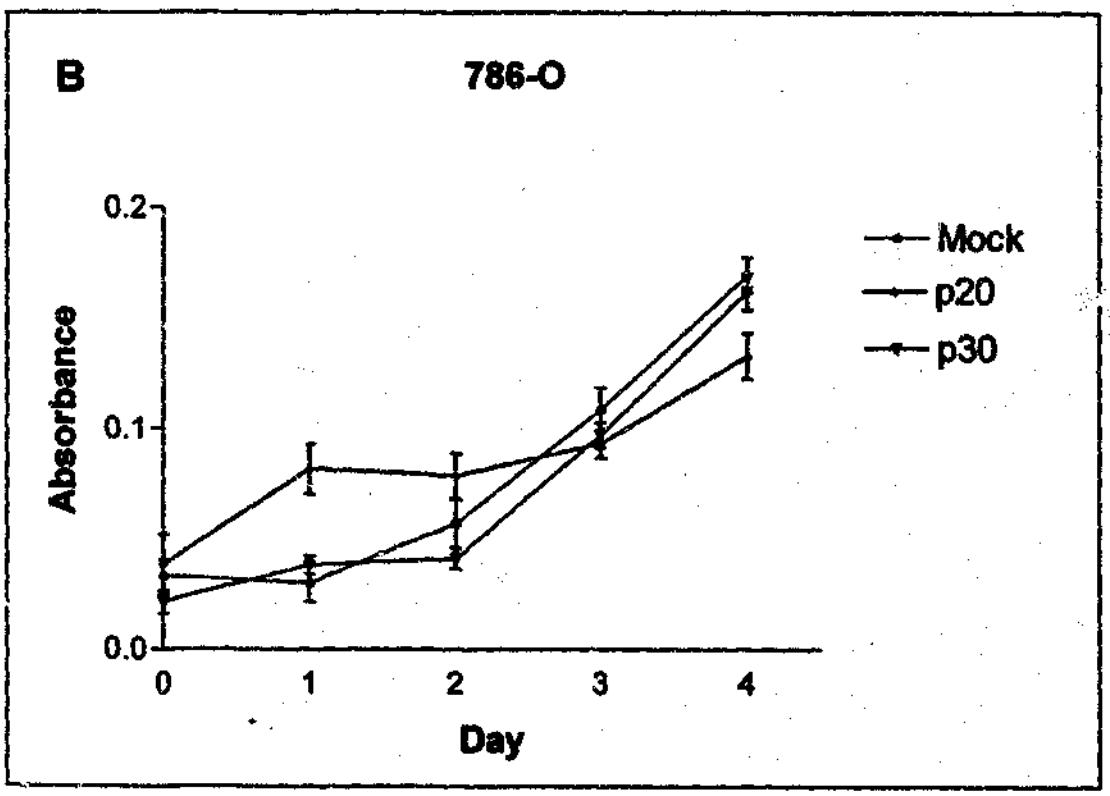


Figure 6.4. Cell growth in a monolayer was measured by MTT colourimetric assay. Mock transfected MCF7 cells showed no significant difference in growth rates compared to ELF5 p20 or p30 expressing cells (A). Mock transfected 786-O cells did not show any significant difference in growth rate compared with *ELF5* p20 or p30 expressing cells (B). Statistical significance was determined by repeated measures ANOVA.

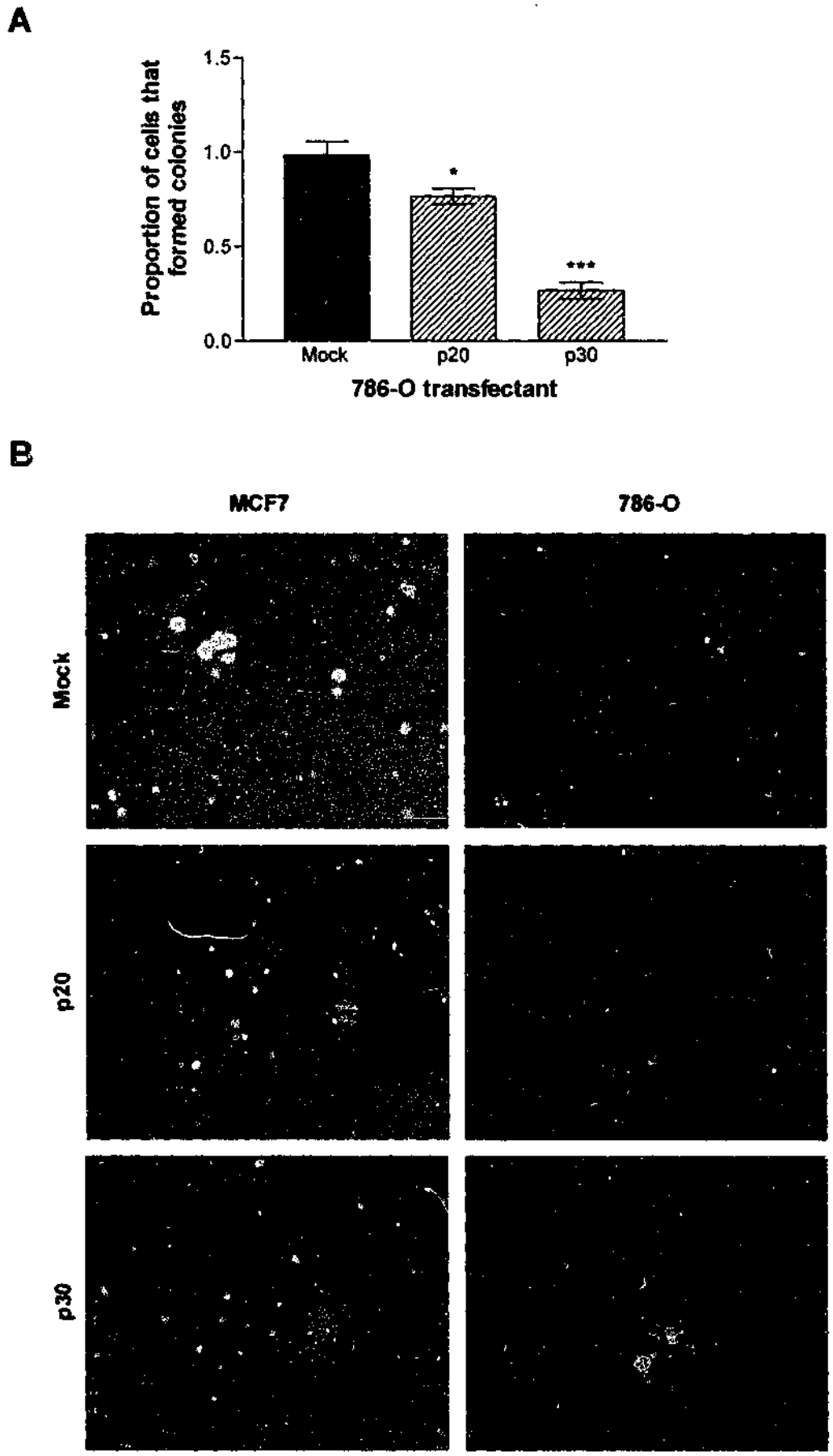


Figure 6.5. Anchorage-independent colony formation of *ELF5*-expressing 786-O renal carcinoma cells is inhibited compared with mock transfected cells. p20 large colony formation is reduced by 22% and p30 by 71% compared with mock transfected cells, where 90% of cells form large colonies (A). n=9;*$p<0.05$; ***$p<0.0001$. Colony size appears to be reduced in p20 and p30-transfected 786-O cells but not in p20 and p30-expressing MCF7 cells (B).

6.3.3 Analysis of *ELF5* expression in primary breast, kidney and prostate carcinoma

Prior to analysing the expression of *ELF5* mRNA in carcinoma samples, the presence of the transcript in normal organs was verified by RT-PCR and hybridisation with an internal oligonucleotide. These data were used to confirm that the organs studied for alterations of *ELF5* expression in carcinoma expressed the transcript in their normal state (Figure 6.8). Breast and skin expressed the transcript at lower levels than the other organs analysed.

ELF5 mRNA expression was then analysed in 38 breast carcinomas by QRT-PCR. One recent publication (Ma et al., 2003a) demonstrated consistently decreased *ELF5* expression in breast carcinomas of various stages compared to normal tissue, but results from our *in vitro* MCF7 study did not show any correlation with potential tumour suppressor activity for *ELF5*. Therefore, breast carcinoma mRNA was obtained to quantify the expression of *ELF5* mRNA in an independent set of samples. Due to the small amounts of material available, not all samples had detectable levels of *ELF5* cDNA, and these were excluded from the study. Graphical representation of the results from 27 informative samples is shown in Figure 6.9. Bars shown in blue or red represent tumour cDNAs that were compared to an unmatched normal breast cDNA, whereas black bars represent samples that were compared to patient-matched normal breast cDNA. Half of the tumour samples had less *ELF5* compared with normal breast and

A

Clone inoculated	Group size	Number of mice that formed tumours
Mock	10	9 (90%)
p20	20	14 (70%)
p30	20	2 (10%)

B

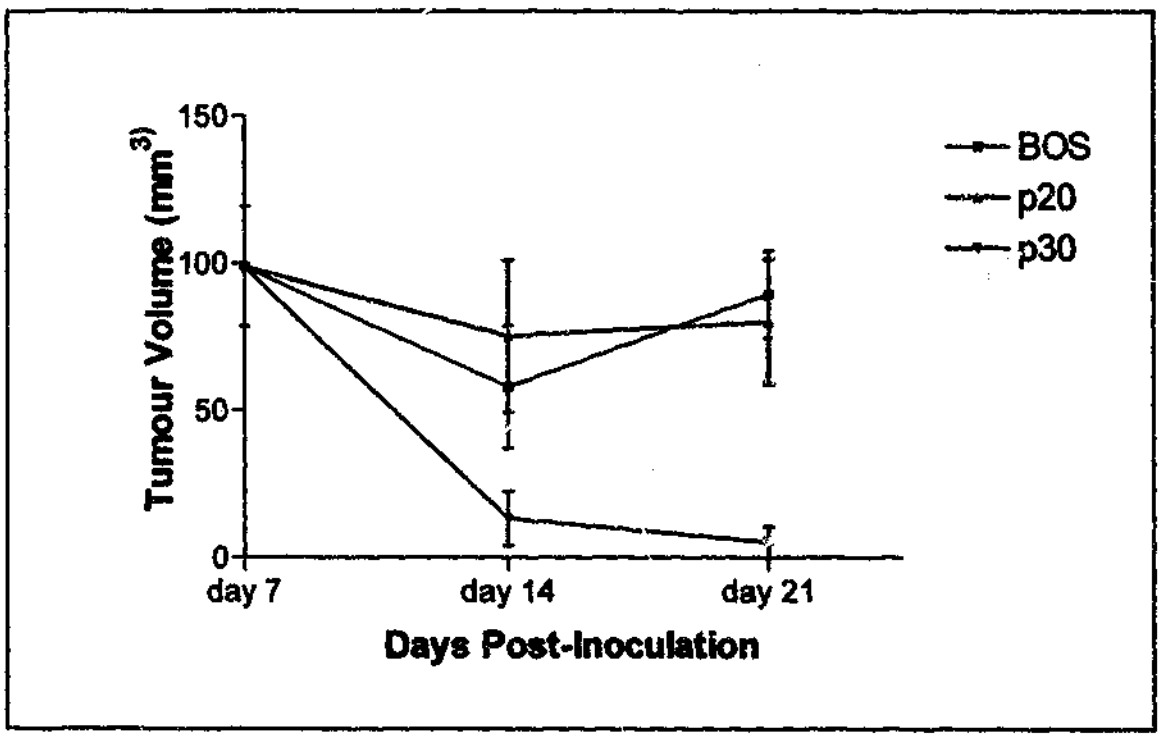


Figure 6.6. Number of inoculated mice that formed tumours at 21 days post-inoculation (A). Group size is cumulative for the number of mice injected over two independent experimental replicates. Results for two p20 expressing clones were equivalent and have been pooled, as have the results for two p30 expressing clones. Average tumour volume per clone type as a function of time post-inoculation (B). the growth curves of mock, p20 and p30 transfected 786-O cells following injection into nude mice are shown. Each data point represents the average tumour volume for 10 mice (mock transfectant) or 20 mice (*ELF5* transfectant).

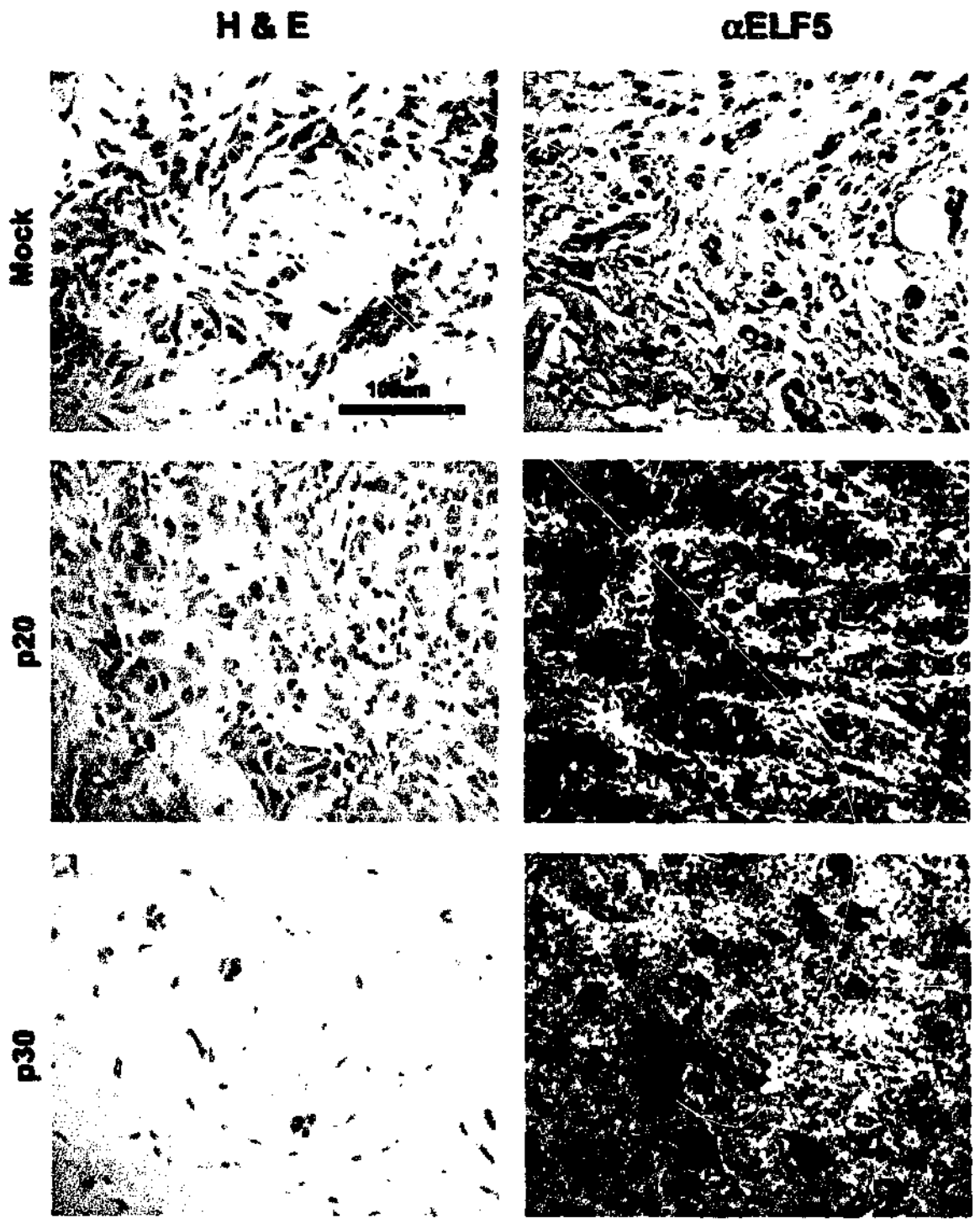


Figure 6.7 Sections of tumours taken from mock, p20 and p30-expressing 786-O SCID mouse tumours show densely packed epithelial-like cells in mock and p20 inoculants, with infiltrating blood cells, but sparse clumps of epithelial-like cells in the subcutaneous fat of p30 inoculants (H&E). Following staining with the anti-ELF5 antibody, clusters of epithelial-like cells are stained intensely in ELF5-expressing cells, but not in the mock transfected cells (αELF5). Brown staining in the subcutaneous fat is evident as is typical for this antibody.

half had an increased amount. These results are not consistent with those reported by Ma *et al.* (2003), who saw a >2-fold decrease in 95 % of their tumour samples. They do not indicate a predictable change in *ELF5* expression in cancerous compared with normal breast, and do not support the hypothesis that *ELF5* is a tumour suppressor in breast epithelium or that its expression is downregulated during cellular transformation.

Expression of *ELF5* mRNA in renal cell carcinomas has not been previously studied, but data from our *in vitro* cell line study suggests that *ELF5* may have tumour-suppressing capacity in renal carcinoma cells. To determine whether this correlates with a loss of expression in primary renal carcinomas, *ELF5* mRNA levels were quantified in 19 renal cell carcinomas compared with normal kidney reference RNA by QRT-PCR. A graphical representation of the results is shown in Figure 6.10. Eighteen of the 19 carcinoma samples showed a decrease in the amount of *ELF5* message detectable, ranging from 5 to >100,000-fold less. Of these 18, all but two samples showed a large decrease (>100-fold). The one cancer sample that had increased *ELF5* expression showed a modest increase only.

Protein expression of ELF5 in breast, kidney and prostate carcinoma samples was examined by immunohistochemistry. Forty-four breast carcinoma and 10 normal breast needle biopsy sections were stained with anti-ELF5 antibody and with pre-immune serum. Results are summarised in tabular form in Figure 6.11, which also depicts representative examples of anti-ELF5 stained carcinoma and normal sections. Overall, the breast carcinomas showed increased intensity of epithelial staining compared with epithelial staining in the normal controls. Some stromal/fat background staining is

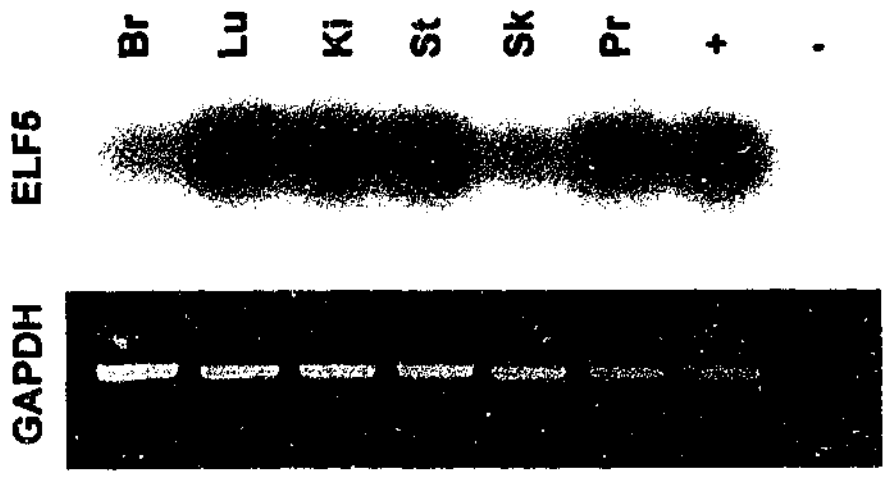


Figure 6.8. ***ELF5* is expressed in a variety of epithelial-rich organs.** *ELF5* expression is shown by RT-PCR and internal oligonucleotide hybridisation, whereas the GAPDH control to confirm the presence of cDNA is shown by agarose gel/ethidium bromide electrophoresis. Br = breast; Lu = lung; Ki = kidney; St = stomach; Sk = skin; Pr = prostate; + = plasmid control and - = water control.

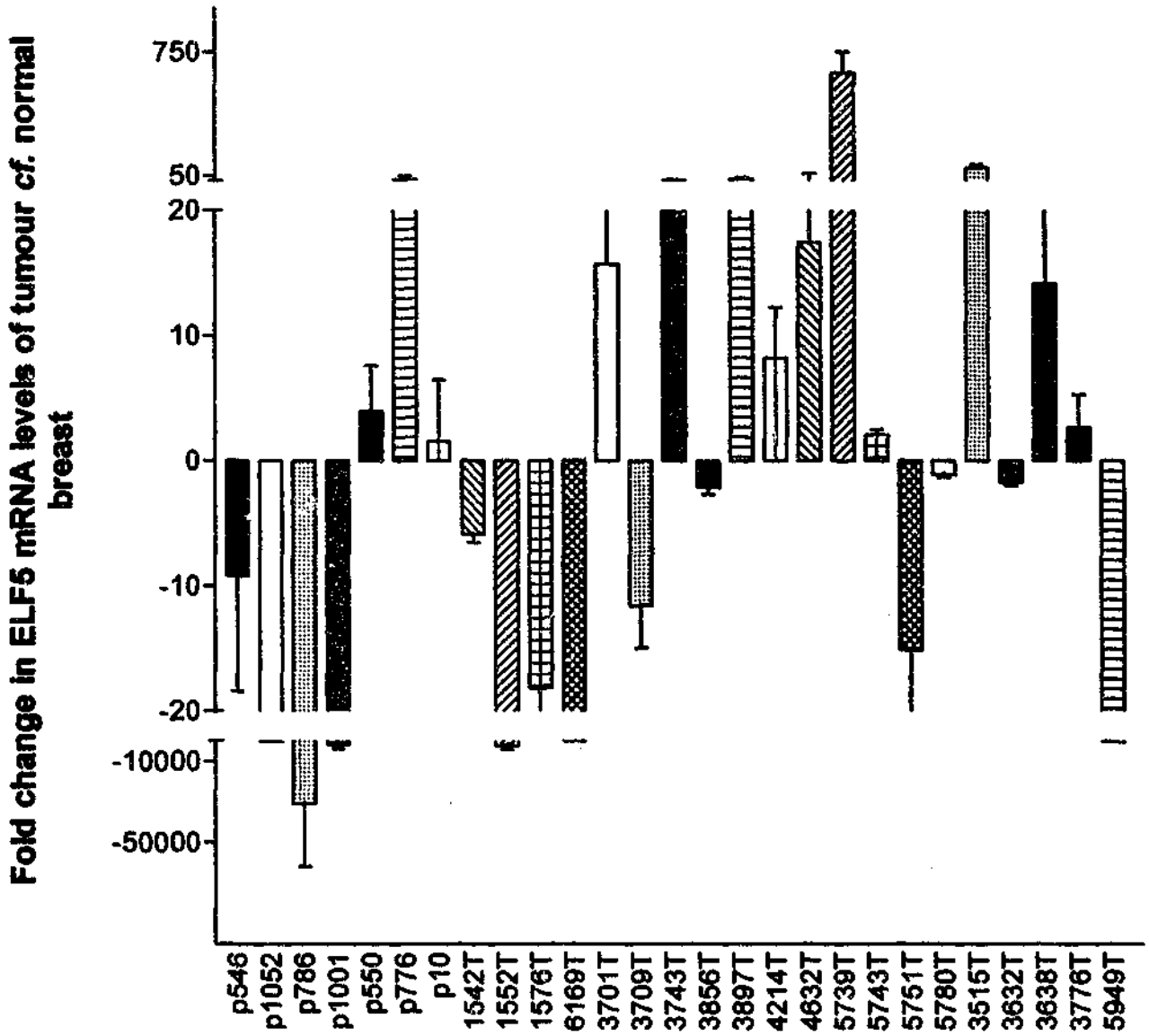


Figure 6.9. *ELF5* transcript levels are changed in breast tumour compared to normal breast samples. Black bars represent samples where the tumour has been compared with a matched normal sample; blue bars represent samples where the tumour has been compared with a reference normal sample as matched normal cDNA was not available. The amount of *ELF5* in each sample was normalised to the amount of *cytokeratin 8*, which represents the quantity of epithelium present; n = 3. Data is presented as fold-change +/- SEM.

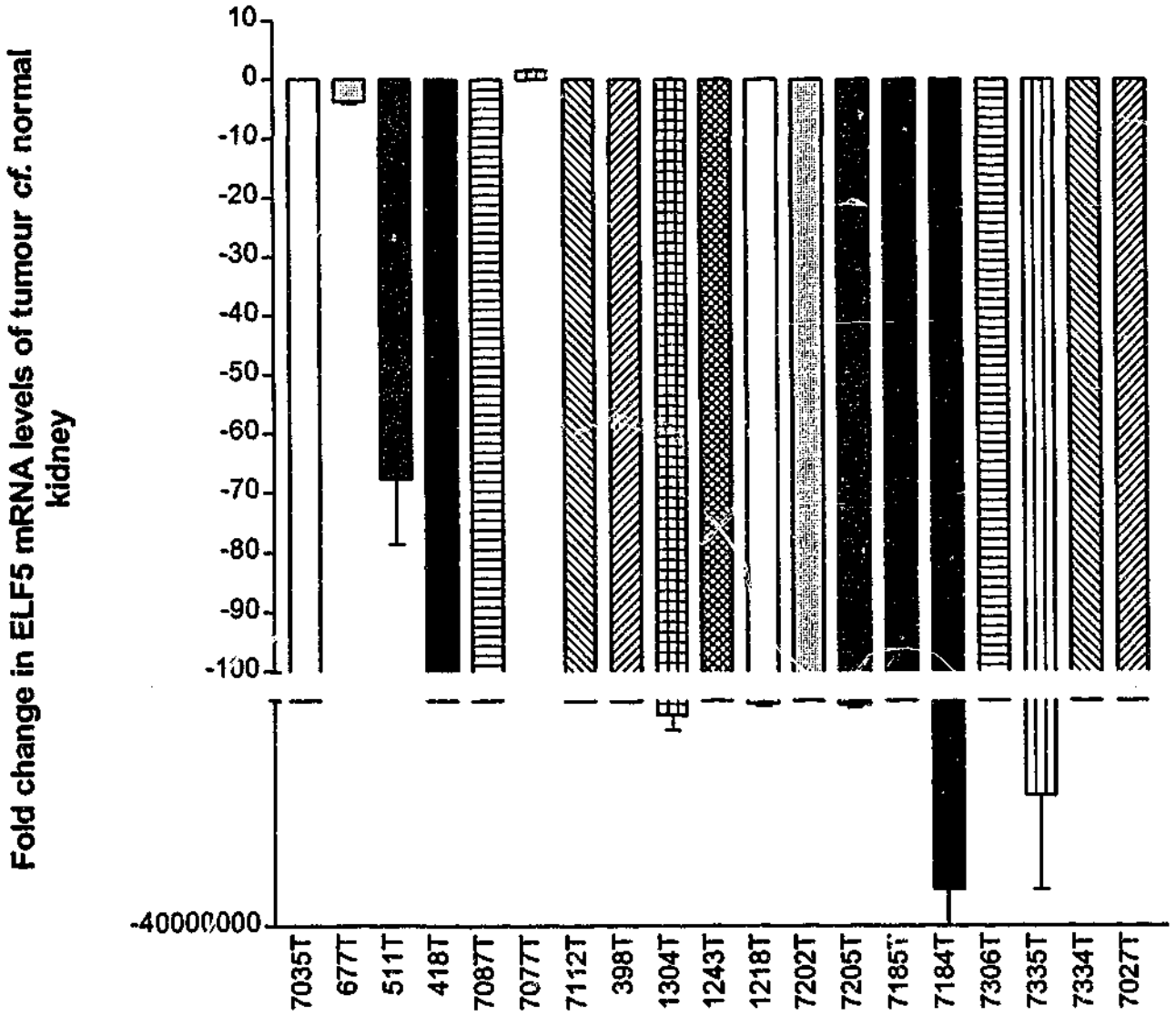


Figure 6.10. *ELF5* transcript levels are decreased in renal cell carcinomas compared to reference normal kidney cDNA. All samples were compared with a reference normal sample as matched normal cDNA was not available. The amount of *ELF5* in each sample was normalised to the amount of *cytokeratin 8*, which represents the quantity of epithelium present; n = 3. Data is presented as fold-change +/- SEM.

evident in sections. This is not ELF5-related staining as shown by adsorption studies, where ELF5-specific antibodies were adsorbed from sera using ELF5 recombinant protein and the resultant serum gave no epithelial staining, but continued to give background on stromal and fatty tissue (data not shown). Increased ELF5 protein staining on primary breast carcinoma is therefore contradictory to the decreased *ELF5* mRNA levels detected in primary breast carcinoma cDNA in half of the samples in this study, and in the study of Ma et al. (2003a), suggesting that there may be an increase in translation of the transcript or that the stability of the protein is different in breast cancerous epithelium compared with normal epithelium.

To determine whether this situation may be unique to the breast, or is perhaps present in other hormone-dependent organs, Qihan Dong surveyed ELF5 protein expression in ten prostate cancers, the ten associated benign prostate hyperplasias (BPH) and in seven normal prostates taken from young accident victims. While no quantification of *ELF5* transcript levels in primary prostate cancers is available, *ELF5* mRNA expression is detectable in hormone-responsive LNCaP prostate carcinoma cells but not in the more aggressive, hormone-refractory, DU145 and PC3 prostate carcinoma cell lines (Oettgen et al., 1999; Zhou et al., 1998), which implies a situation that has parallels to that of the breast. No ELF5 staining was detectable in normal prostate sections, but cancerous sections showed weak to strong staining, while associated BPH showed no to weak staining (Figure 6.12). In the two cancer cases with weak ELF5 staining, the BPH showed no staining. In the other eight cancer cases with moderate *ELF5* staining, the BPH showed weak staining. This indicates that while *ELF5* mRNA may be expressed at low levels in the normal prostate (Figure 6.8), it is either not translated into a functional protein product or the protein product is not abundant enough to detect in our

assay. In hyperplastic prostates some translation occurs as the protein is detectable, but as judged by staining intensity, the amount seems to be increased in cancer.

ELF5 protein and mRNA expression was studied in the hormone-independent kidney with results that contrasted to those of the breast and prostate. While *ELF5* mRNA is expressed in the normal kidney (Figure 6.8) and the protein is detectable by immunohistochemistry in normal kidney, ELF5 protein is undetectable in kidney-derived carcinomas (Figure 6.13). These results concur with the loss of *ELF5* mRNA expression in kidney-derived carcinomas, and indicate that the loss of protein is due to a decrease in transcription rather than translation.

6.4 Discussion

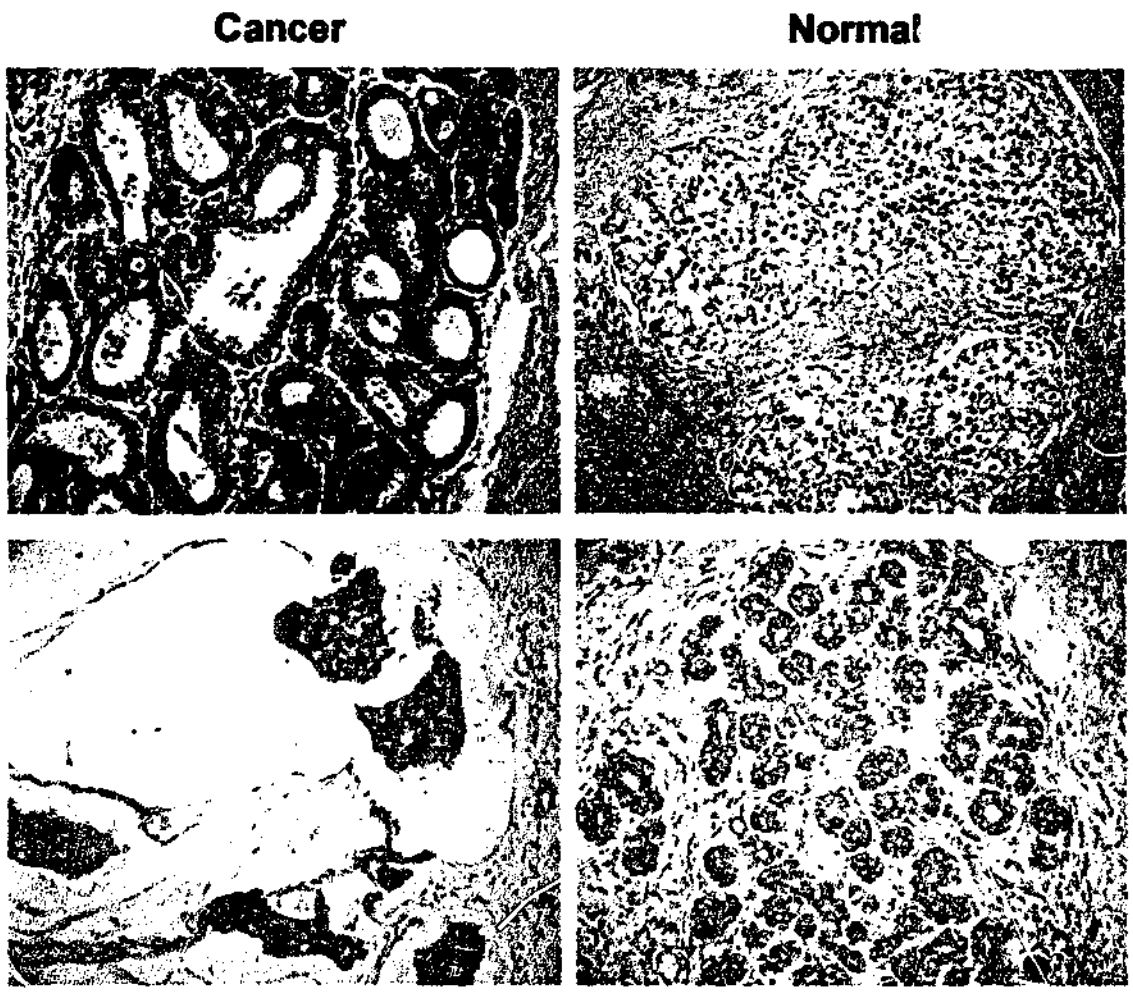
6.4.1 *ELF5* does not display the characteristics of a tumour suppressor gene in breast epithelium

ELF5 mRNA is expressed in epithelial cells found in a restricted subset of organs. In this study, *ELF5* mRNA is barely detectable in the normal human breast, though it has been detected more readily in other studies (Ma et al., 2003a; Oettgen et al., 1999). This may relate to the source of RNA, which was derived from small tissue biopsies taken during tumour resection surgery. It is also possible that *ELF5* is induced in the breast via the hormonal response, and that detectability of the transcript could depend on the state of the breast when the sample was taken. However, a recent review of the available literature noted that *ELF5* was expressed at low levels in the normal breast (Feldman et al., 2003b).

The circumstantial data surrounding *ELF5*: that is, its chromosomal localisation at the tumour suppressor-rich locus 11p13; loss of expression in epithelial-derived cancer cell lines and its relationship to the *ets* oncogene/tumour suppressor gene family, led to the hypothesis that *ELF5* would exhibit characteristics of a tumour suppressor gene in the normal versus the cancerous epithelium of organs it is expressed in. The primary organ of interest was the breast, given that *ELF5* mRNA expression appeared to be decreased in breast cancer cell lines (Zhou et al., 1998) and in a set of primary breast carcinomas of various gradings (Ma et al., 2003a). In addition, the expression of *ELF5* is a critical requirement for murine pregnancy-associated mammary gland development, indicating a unique, non-redundant role for this gene in the mammary gland (Zhou et al., PhD Thesis, 2001; Appendix VII). None of our investigations into the function of *ELF5* in breast cancer suggested that it had tumour suppressor activity in this organ. There was no genomic loss of an allele in the 18 breast tumours tested, and QRT-PCR profiling of 27 breast cancers compared with normal breast epithelium showed aberrant expression of the transcript, but not a change that was consistent either in direction (increase or decrease) or magnitude. Half of the cases had increased *ELF5* expression and half decreased, which conflicts with a study on a separate cohort where 7/8 ADH, 28/30 DCIS and 25/25 IDC (overall 95%) tested had decreased *ELF5* expression (Ma et al., 2003a). Four of the 27 informative cases profiled by QRT-PCR in this study were also tested for LOH. None had undergone LOH, but three had small increases in *ELF5* mRNA levels in tumour compared with normal breast epithelium, while one had decreased expression in the tumour. With such small numbers, these data do not indicate a tendency for cases that lack LOH to have increased *ELF5* mRNA, though this possibility cannot be discounted.

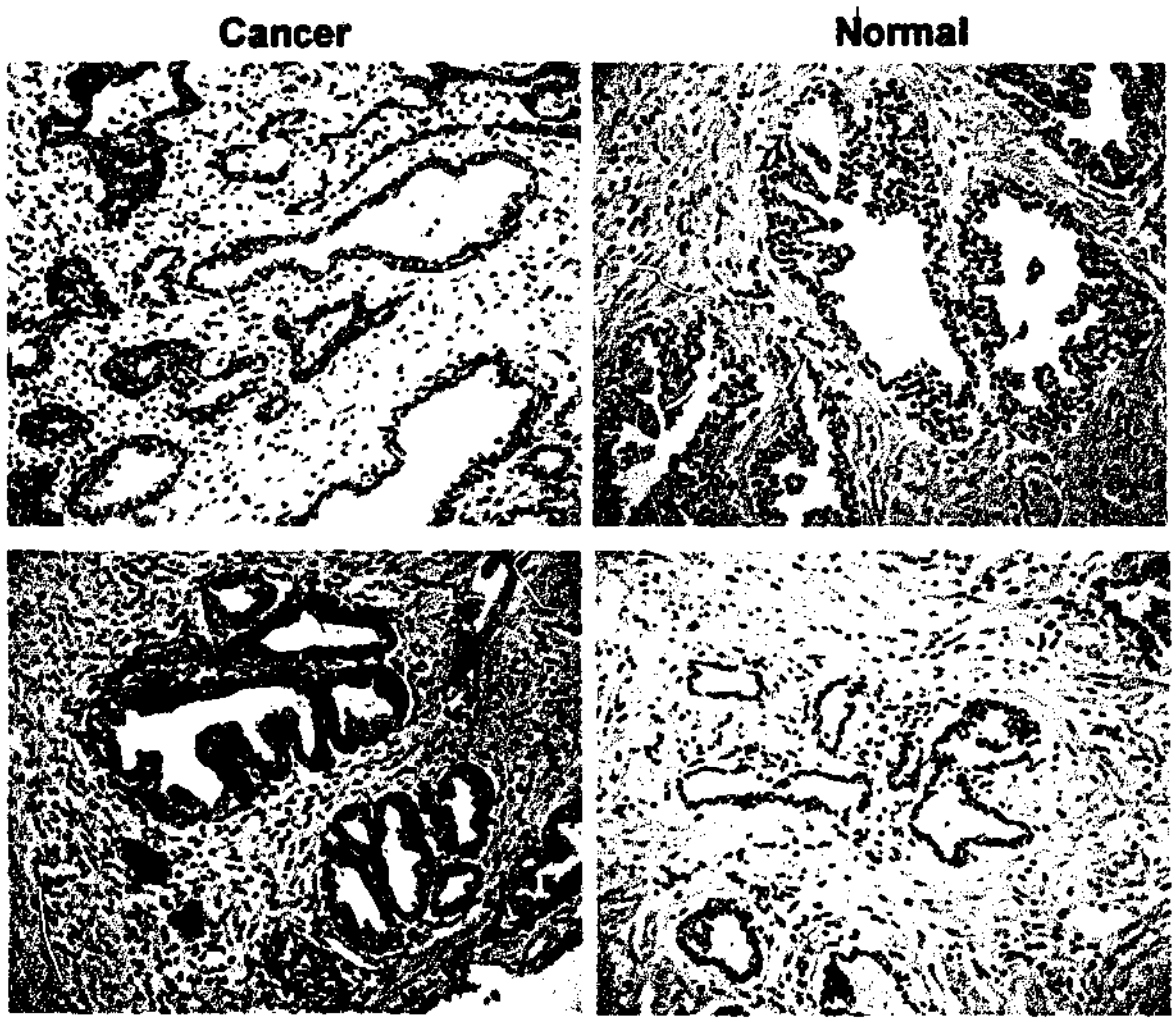
At the protein level, 44 breast carcinomas, 34 of which were infiltrating ductal carcinomas and 10 of which were metastases to lymph nodes, retained and apparently had increased ELF5 expression. Re-introduction of *ELF5* into non-expressing MCF7 breast cancer cells had no effect on growth, either when the cells were attached to a plate or when they were growing in a three-dimensional anchorage-independent state, which is one of the capabilities that epithelial cells gain only following transformation. Collectively, these data indicate that *ELF5* expression is not consistently decreased in breast cancer and that the gene is unlikely to act as a tumour suppressor in the context of the breast.

Within any one case, only mRNA *or* protein expression was tested as the samples were obtained from different sources. Insufficient tissue was available to perform multiple tests on each case. Therefore, direct correlations could not be drawn between the level of mRNA and protein expression. In further work, comparing mRNA expression levels with protein expression levels by Western blotting may prove informative by indicating whether alterations in translation of *ELF5* occur in the carcinoma. This may address the paradox of why, although transcript levels can be either increased or decreased in breast carcinoma, the strong immunohistochemical staining is so consistent. No other studies have yet reported on the protein expression of *ELF5* in cancer, making these data the first to suggest that ELF5 protein may be dysregulated in breast carcinoma.



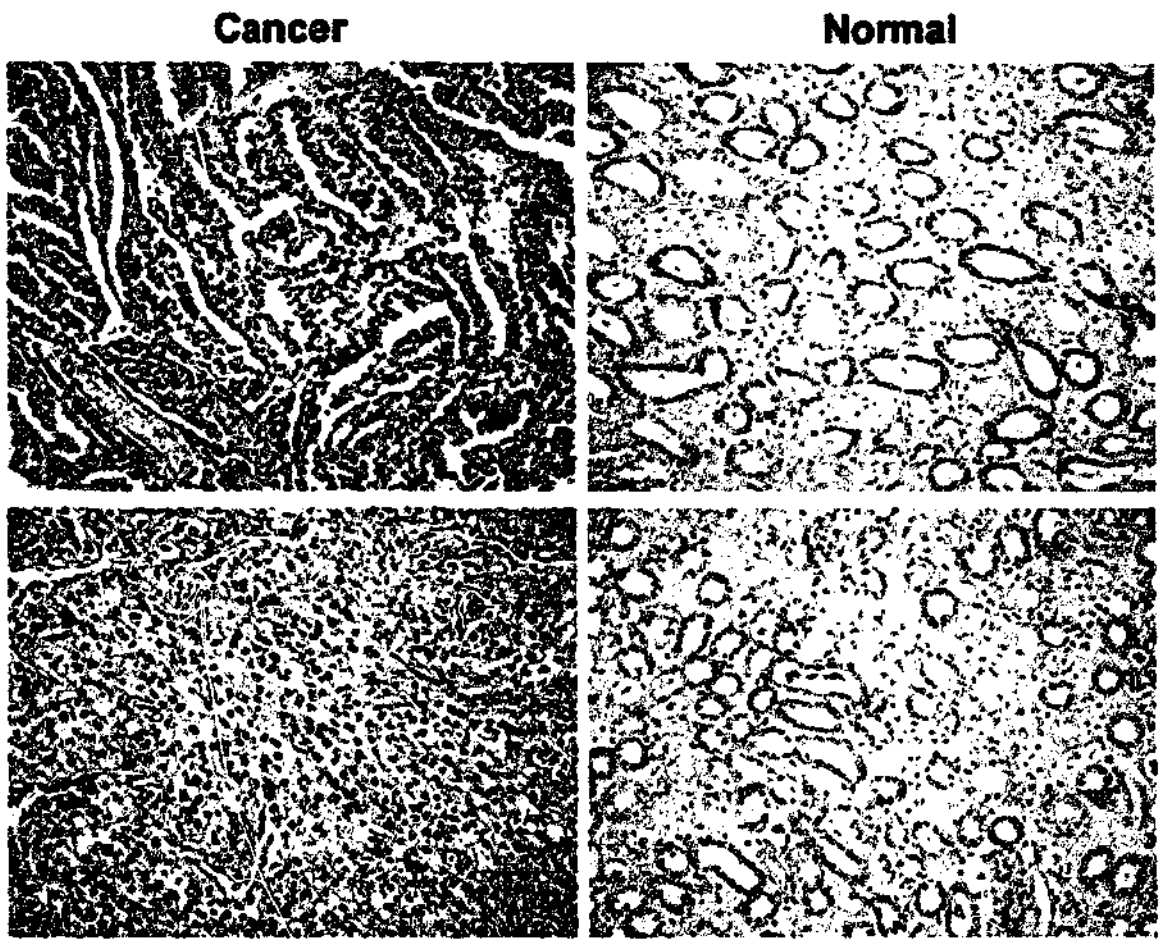
Sample Type	Number of Samples	Epithelial Staining Intensity
Carcinoma	44	+++
Normal	3	+
Normal	7	-

Figure 6.11. Immunohistochemical analysis of ELF5 in breast carcinoma. Two carcinomas showing intense epithelial staining for ELF5 in abnormal ducts are representative of the cancer sample population. Two normal breasts showing no to weak staining for ELF5 in normal epithelial structures are representative of the normal sample population. Results for each sample are summarised as: +++ = strong staining; ++ = moderate staining; + = weak staining; - = no staining. Magnification: 200X.



Sample Type	Number of Samples	Epithelial Staining Intensity
Cancer	8	++
Cancer	2	+
Associated BPH	10	+/-
Normal	7	-

Figure 6.12. Immunohistochemical analysis of ELF5 in prostate carcinoma. Two carcinomas showing epithelial staining for ELF5 in abnormal ducts are representative of the cancer sample population. Two normal prostates showing no staining for ELF5 in normal epithelium are representative of the normal sample population. Results for each sample are summarised as: +++ = strong staining; ++ = moderate staining; + = weak staining; - = no staining. Magnification: 200X. Immunohistochemical staining and interpretation provided by Qihan Dong.



Sample Type	Number of Samples	Epithelial Staining Intensity
Cancer	40	-
Normal	9	++

Figure 6.13. Immunohistochemical analysis of ELF5 in kidney carcinoma. Two carcinomas that lack epithelial staining for ELF5 in abnormal kidney epithelium are representative of the cancer sample population. Two normal kidneys showing staining for ELF5 in normal kidney epithelium are representative of the normal sample population. Results for each sample are summarised as: +++ = strong staining; ++ = moderate staining; + = weak staining; - = no staining. Magnification: 200X.

The data from this study as a whole do not contradict the possibility *ELF5* could still be associated with cancer in the breast, but as an oncogene. It is expressed strongly in the growth and differentiation phases of pregnancy-associated mammary gland development and, via involvement in the prolactin response pathway, promotes one or both of these cellular programmes (Harris et al., submitted). Other growth-promoting elements in the prolactin response pathway have been found to be over-expressed and potentially oncogenic in breast cancer, including prolactin, cyclin D1, IGF-2 (Brisken et al., 2002; Brockman et al., 2002) and CPAP (Peng et al., 2002); *ELF5* may be another of these. A different set of experiments will be needed to test this hypothesis, including the introduction of *ELF5* into a non-cancerous cell line to identify whether it has transforming activity, an experiment that has been performed on a litany of oncogenic *ets* factors (Buijs et al., 2000; Chan et al., 2003; Deneen and Denny, 2001; Hart et al., 1995; Kaya et al., 1996; Seth and Papas, 1990).

6.4.2 *ELF5* may not act as a tumour suppressor in the prostate

While the data concerning the expression of *ELF5* in the normal prostate, prostate cancer and associated BPH is less complete, the protein is detectable in the cancer and to a lesser extent BPH, but not in normal prostate. This contrasts with the positive expression of *ELF5* mRNA in normal prostate but loss of expression in the hormone-independent prostate cancer cell lines PC3 and DU145 (Zhou et al., 1998). This situation appears to be similar to that seen in the breast and associated breast carcinoma, as discussed above. If ELF5 promotes the proliferation of prostate and breast epithelium, there may be a selective advantage for pre-cancerous cells to upregulate production of the protein product. Expressing deletions of the *ELF5* transcript in cell lines and analysing their translation into protein may assist in determining whether the

5′ or 3′ UTRs of *ELF5* have a function in translational regulation. The use of co-immunoprecipitation and Y-2-H analysis to place *ELF5* in an intracellular signalling pathway, to recognise the factors it responds to and those it proceeds to activate, will assist in determining the role *ELF5* plays in normal epithelial growth and differentiation and therefore in understanding its activity in carcinoma formation.

6.4.3 *ELF5* expression is decreased in kidney carcinoma, and re-expression of the gene in renal carcinoma cells suppresses growth and tumourigenicity

Renal cell carcinoma is an increasingly important clinical disease – apparent incidence has increased due to improvements in detection, but treatment has not made equivalent progress, and the aetiology of the disease is not well understood. Kidney cancer causes mortality of 3 per 100,000 (women) and 7 per 100,000 (men) in Australia (Department of Epidemiology and Preventative Medicine, Monash University; Department of Nephrology, Monash Medical Centre; Report to Kidney Foundation, 2000).

Increased risk of kidney cancer is associated with specific diseases such as kidney infection and diabetes type I and II (Schlehofer et al., 1996). There is an unidentified genetic component, with an increased relative risk of the cancer if a first-degree relative has also been diagnosed with kidney cancer (Schlehofer et al., 1996).

In the kidney, *ELF5* mRNA is drastically decreased in 95% of the carcinomas studied. This is consistent with the lack of immunoreactivity of the *ELF5* antibody on kidney-derived carcinomas, whereas the normal kidney stains strongly throughout the tubule. Re-expression of *ELF5* in renal carcinoma cells results in decreased three-dimensional, anchorage-independent growth and in tumourigenicity *in vivo*. p30 injection sites

where the kidney-derived cells are present seem to lack tumour-associated blood vessels and blood cell infiltrations, which are characteristics of aggressive tumours that initiate formation of their own blood supply. Though statistically significant in both cases, the effect of decreased tumourigenicity for *ELF5*-expressing 786-O cells is minor for the p20 isoform of *ELF5* but much greater for the p30 isoform. This seems logical as p30 is the only *ELF5* isoform detectable in normal kidney (unpublished data).

In the kidney, *ELF5* possesses the characteristics of a functional tumour suppressor gene. Determining the mechanism by which it acts will require further study and access to reagents. Access to kidney carcinoma derived genomic DNA and matched PBL DNA is necessary to investigate LOH at the *ELF5* locus in this carcinoma subtype. If LOH is not occurring, the DNA could also be used to investigate promoter hypermethylation as an alternative mechanism for gene silencing. Somatic mutation in the gene resulting in transcript instability could also account for the loss of expression, but is less likely to be a mechanism common to all cases. Ascribing a purpose for *ELF5* in normal kidney development and maintenance of function using the techniques described above will assist in elucidating how dysregulation of this tumour suppressor gene can result in one, or any, of the various phenotypes of the cancerous cell.

6.4.4 Conclusions

The activity of *ELF5* in cancer depends on the function it has in the organ from which the cancer was derived. In the hormone-dependent development of the breast and prostate, *ELF5* must interact with different signalling pathways to those it does in the hormone-independent kidney. These roles may be to do with growth promotion or suppression, or with terminal differentiation of epithelial cells. In the breast, *ELF5* may

promote cellular proliferation via its participation in the prolactin response pathway, as it is a master regulator controlling several arms of the prolactin pathway endpoints. No data is available on the activity of the protein in the prostate. In skin-derived keratinocytes *ELF5* expression increases during the terminal differentiation process as the cells stop proliferating (Oettgen et al., 1999); speculatively, this situation may be paralleled in the kidney. Other work in this thesis has shown that *ELF5* can act as either a transcriptional activator or repressor in different promoter and cell type contexts (Chapter 5). This indicates that its biological activity as an inducer of proliferation or of terminal differentiation is dependent on both these contexts, and on the different responses to extracellular signals required in different organs. Dissection of the intracellular signalling pathways that *ELF5* acts in, and the discovery of target promoters that it transactivates or transrepresses in normal organ development will provide more information on why this gene is a context-dependent tumour suppressor.

Chapter 7. Discussion and Conclusions

This study aimed to define the protein expression pattern of the *ets* transcription factor *ELF5* in the mouse, to elucidate potential transcriptional regulators of the gene and to investigate its function as a transcription factor relative to the process of human epithelial carcinogenesis.

7.1 Expression of Elf5 is epithelial-specific and controlled at transcriptional, translational and post-translational stages

Initial characterisation of murine and human *ELF5* expression utilised whole organ lysates and Northern analysis. *ELF5* was presumed to be epithelial-specific as the transcript was detected in organs that contained high proportions of epithelial cells (Oettgen et al., 1999b; Zhou et al., 1998) and due to its close evolutionary relationship with *ELF3*, another *ets* gene expressed in epithelial-rich organs (Oettgen et al., 1999a; Tymms et al., 1997). Production of an ELF5-specific antibody allowed immunohistochemical localisation of the protein to epithelial cells of a subset of murine organs in which the mRNA had been detected. While the transcript was detected in murine lung, bladder, kidney, skin, stomach, mammary gland, prostate and salivary gland, the protein was detectable by immunohistochemistry in the epithelium of the kidney, mammary gland, salivary gland and

stomach only. Elf5-specific staining was confined to simple glandular epithelial structures, confirming the initial presumption that it is an epithelial-specific protein.

The discrepancy between transcript and protein expression of Elf5 indicates that in normal murine tissues the gene is being regulated at both transcriptional and translational levels. This process is probably also applicable to the human context, as the transcript was detected in the same array of human tissues as in the mouse, by RNA dot blot analysis (Oettgen et al., 1999b), but the protein was only detectable by immunohistochemistry in breast (Chapter 6), stomach (data not shown) and kidney (Chapter 6). Salivary gland was not examined due to lack of availability of the tissue. The protein was undetectable in normal prostate. Transcriptional regulation of ELF5 in the human context is discussed later (Section 7.2).

There is a paucity of literature that comparatively examines the spatial expression patterns of *ets* factor mRNA and protein. Early studies examined *ets* expression by Northern analysis due to the lack of antibodies for protein-based detection methods (Klemsz et al., 1993; Kola et al., 1993; Queva et al., 1993; Ray et al., 1992; Schuur and Baluda, 1991; Su et al., 1996; Watson et al., 1992). Later studies have used antibodies in immunohistochemistry and Western analysis for comparing the expression between cancer and the normal tissue from which it is derived, rather than surveying protein expression in a variety of normal organs (Baert et al., 1997; Jobling et al., 2002; Kanda et al., 2002). This type of work has presumably been delayed because of the time taken to produce specific

antibodies directed against non-conserved regions outside the *ets* domain, and because of the scarcity of normal human tissue as a resource.

Dysregulation of transcriptional and translational control of the *ets* factor, *PSE*, has been noted in cancer. This does not appear to have been studied in the case of other *ets* genes. PSE protein is expressed in prostate, colon and breast. It is reduced in invasive breast carcinoma and absent in breast carcinoma cell lines, although the mRNA is upregulated (Feldman et al., 2003a; Ghadersohi and Sood, 2001). The mRNA is translated in normal but not cancerous prostatic epithelium (Nozawa et al., 2000), so this translational control does not seem to apply to differential PSE mRNA and protein expression in non-transformed cells.

When investigating *ets*-dependent gene regulation, many studies have preferentially, and rightly, investigated a functional response: that is, the amount of target gene transcription, alteration in phosphorylation state of the transcription factor in response to a stimulus, or DNA-binding activity (Paumelle et al., 2002); reviewed in (Fujimura et al., 1994; Goel and Janknecht, 2003; Yordy and Muise-Helmericks, 2000). However, this approach may miss more subtle levels of regulation. For instance, the presence of a specific mRNA within a cell, but not the corresponding protein, may indicate that translation of the factor is rapidly required after exposure of the cell to a certain environmental stimulus. Investigating this possibility could help explain both the reason for, and the mechanism by which the expression of a factor is controlled within the cell, and help to understand the complex regulation of factors such as *ELF5*.

Little is known currently about which stimuli, or signalling cascades, may induce activation of *ELF5*. Preliminary work has been carried out suggesting that *ELF5* integrates prolactin signalling in the mammary gland (Appendix VII) but these data are limited to analysing the up- and down-regulation of *ELF5* mRNA levels in murine pregnancy-associated mammary gland development. Bacterially-produced full-length recombinant *ELF5* does not specifically bind DNA (Chapter 5) but requires truncation at the N-terminus. To carry out its function in development, ELF5 presumably must be modified post-translationally, similarly to other *ets* factors. *Ets* proteins are targets for dephosphorylation by signalling pathways including various arms of the MAPK pathway (Ets-1 (Bradford et al., 1995), Net (Giovane et al., 1994), Elk-1 (Chuang and Ng, 1994; Galetic et al., 2003; Swiatkowski et al., 2003), Ets-2 (McCarthy et al., 1997; Pestell et al., 1996), ER81 and ERM (Janknecht, 1996; Janknecht et al., 1996) and ESE-3 (Tugores et al., 2001)); the JAK-STAT pathway (PU.1 and Spi-B (Nguyen and Benveniste, 2000) and Ets-2 (Rameil et al., 2000)) and the Akt (protein kinase B) signalling pathway (Elk-1 (Galetic et al., 2003) and Ets-2 (Smith et al., 2000)). Discovering which pathways post-translationally modify ELF5 and hence control its activity will help illuminate the role that ELF5 plays in development.

Evidence from this study indicates that *ELF5* is being actively regulated at various levels. Firstly, it is transcriptionally regulated to ensure specificity of expression within a subset of glandular epithelium, and it is temporally regulated throughout murine mammary gland development (Zhou et al., 1998). NFκB and STAT1 have been identified as potential regulators of the promoter. Secondly, translation of the transcript is regulated, permitting

detection of the protein only within a proportion of the organs in which the transcript is detectable. Thirdly, it must be post-translationally modified to become a functional transcription factor, as truncation of the N-terminus of the protein is required for DNA-binding activity *in vitro* (Oettgen et al., 1999b). These data are among the first to imply that *ELF5* is regulated, like other *ets* factors, at the translational and post-translational level. Further work focusing on identifying protein kinases, or proteases, that modify ELF5 in response to intracellular signalling cascades will be a step forward in discovering what the purpose of this transcription factor is in normal development, and in cancer.

7.2 Transcriptional regulation of ELF5 involves NFκB and STAT family transcription factors

Elf5 is transcriptionally regulated in a temporal manner throughout murine mammary gland development. Given that the gene sequence is highly conserved between human and mouse genomes, a similar situation may be occurring in the human breast although there is currently no information available on this. However, transcriptional control of *ELF5* differs between the human and murine genes. The murine gene has two alternative splice isoforms that differ by use of alternative 3′ UTRs, whereas the human gene has three transcriptional isoforms that differ in the coding region but utilise the same 3′ UTR: p20, p30 and ESE-2a.

Therefore, though the coding sequence of *ELF5* is under heavy selective pressure, resulting in only one non-conservative amino acid change between mouse and human proteins, the regulation of the gene has evolved during speciation.

The differences in gene regulation that exist between the human and mouse loci may reveal a more complex role for the protein in higher mammalian organisms – one determined by more sophisticated gene regulation, rather than by the limited coding sequence variation that exists between murine and human genes. Particularly in the mammary gland, divergence in promoter sequence of growth- and differentiation-associated genes allows for non-parallel mechanisms of pregnancy-associated mammary gland development between species: that is, the murine mammary gland behaves differently from the human gland in pregnancy-lactation-involution cycles, and this difference may be caused by changes in gene regulation during these cycles, although the genes involved are the same.

The human *ELF5* promoter contains a variety of predicted transcription factor recognition sites that may be relevant to epithelial-specific *ELF5* transcriptional regulation. These include EBS, NFκB, STAT, AP-1, deltaEF1, USF and OCT1 sites, some of which are evolutionarily conserved between human and murine loci (AP-1, OCT1 and NFκB sites; R. Chehab, unpublished data). The EBS sequence is compatible with preferential binding by epithelial-specific *ets* factors including *ELF5* and *ELF3*, but neither of these proved to have any effect on promoter activity. Few examples of *ets* transcriptional autoregulation are available in the literature. The myeloid-specific factor, PU.1, binds to a tissue-specific element within its own promoter, creating a positive autoregulatory loop (Chen et al.,

1995). Also, in NIH3T3 cells, exogenous Ets-1 positively regulates the endogenous *Ets-1* promoter (Majerus et al., 1992; Oka et al., 1991; Seth and Papas, 1990). More frequently, *ets* factors are transcriptionally regulated by other *ets* proteins. Both human and murine *Fli-1*, where the regulatory regions are highly conserved, are regulated combinatorially by GATA-1 and another B-cell specific *ets* transcription factor, Spi-1 (Barbeau et al., 1999). Due to the incomplete understanding of the selectivity of ets proteins for binding to particular motifs, it could be that almost any of the other *ets* factors available in epithelial cells is the factor requisite to act through this motif.

NFκB is a gene of interest regarding the potential role of *ELF5* as a tumour suppressor in breast and prostate cancers. NFκB also has an established presence as a regulator of lobuloalveolar proliferation, ductal side branching and differentation during murine mammary development (Brantley et al., 2001). It associates with *ets* factors on the promoters of downstream target genes, in the myeloid cellular context (Gri et al., 1998; Lodie et al., 1998; Thomas et al., 1997). For example, in plasma cells, NFκB and a nuclear *ets* factor are both critical for transcriptional enhancement activity via the immunoglobulin heavy chain 3' enhancer after ligand-receptor induced stimulation of resting B cells. The factors co-operatively bind a paired NFκB/ets motif and are both essential for transcriptional enhancement (Linderson et al., 1997).

NFκB is over-expressed in breast and prostate cancers (reviewed in Rayet and Gelinas, 1999). Its activity in these cancers relates to increased cellular growth rates, resistance to apoptosis (Abdel-Mageed and Agrawal, 1998), metastatic capability (Denoyelle et al.,

2001) and drug resistance (Gu et al., 2002). Although the selective advantages to the cancer cell with increased NFκB activity are clear, the mechanism by which NFκB activity results in these endpoints is not. Perhaps the transcriptional downregulation of *ELF5* is part of one of these pathways.

Many instances have been noted where ets/NFκB interactions are required for target activation (reviewed in Li et al., 2000). It is feasible that the promoter regulation of *ELF5* by NFκB is part of a feedback loop, where upon a certain cellular stimulus, ELF5 and NFκB are activated to interact on downstream target promoters. Increasing levels or activation states of NFκB could then result in a negative feedback loop that represses ELF5 production, attenuating the response. Little is known about the mechanisms of *ELF5* interaction with other proteins and its activity on target promoters, but current studies underway involving Y-2-H and production of *ELF5*-specific monoclonal antibodies to perform chromatin immunoprecipitation (ChIP) and co-immunoprecipitation (co-IP) assays should produce an answer to the negative feedback loop hypothesis.

STAT proteins are excellent candidate regulators of *ELF5* regarding both mammary gland development and carcinogenesis. While in T47D cells only STAT1 has a significant effect on the *ELF5* promoter, it is possible that in non-transformed mammary cells this situation could be different. The PRL-JAK-STAT response pathway is not complete in T47D cells, as they do not functionally differentiate to produce milk proteins in response to PRL, which is the pathway endpoint. In a functional mammary epithelial cell context, or following stimulation by other growth factors, it is possible that other STATs regulate *ELF5*.

STATs 1, 3 and 5 are all expressed in mammary epithelium and are established as tumour suppressors or oncogenes in the context of this organ. STAT1 is activated in a subset of breast cancers, and correlates with improved relapse-free survival after treatment (Widschwendter et al., 2002). While it is over-expressed in a proportion of breast cancers, its presence is associated with an improved prognosis, probably due to its role in interferon (IFN)-mediated growth suppression.

Activated NFκB and STAT both interact with the interleukin-6 (IL-6) signalling pathway. The IL-6 pathway could provide a point whereby both NFκB and STAT are integrated to alter ELF5 expression. IL-6 is a pro-inflammatory, pro-malignant cytokine (Neumark et al., 2003), and it acts as a paracrine growth factor that inhibits the growth of ER+ breast cancer cell lines but not ER- cell lines, which could translate *in vivo* to a selective pressure for more aggressive, estrogen-insensitive tumours (Chiu et al., 1996). NFκB induces the pleiotropic cytokine, IL-6, after a variety of signals that include oxidative stress (Zhang et al., 2001), stimulation by factors such as insulin-like growth factor II (IGFII) (Kwon et al., 2000) and interaction with drugs and cell surface molecules. In intestinal epithelial cells, the presence of IL-6 induces NFκB release from IκB, allowing NFκB translocation to the nucleus (Wang et al., 2003). STAT3 and STAT1 are phosphorylated following cell exposure to IL-6 via the gp130 cell surface receptor and transactivate downstream target genes (Coffer et al., 1995; Zhang et al., 1995b; Zhong et al., 1994). The NFκB and STAT studies have, however, been performed mainly in blood, hepatic and intestinal cells, not in mammary cells or tissue.

Breast tumours may be divided into groups based upon gene expression. Some tumours over-express NFκB, or IL-6, or STAT1, but data on expression of all three genes as a group is not yet available. Tentatively, those that over-express NFκB, and therefore IL-6, may be deficient in ELF5. Those that over-express or have constitutively active STAT1, may also increase transcription of ELF5, accounting for the two populations, differing in ELF5 expression status, that were observed in our study. The publicly available microarray data on gene expression in breast cancers (Bertucci et al., 2002a; Bertucci et al., 2002b; Bertucci et al., 2003; Ellis et al., 2002; Gu et al., 2002; Inoue et al., 2002; Perou et al., 1999; Sorlie et al., 2003; Wang et al., 2002) was investigated to find evidence that bears on this hypothesis, but none had expression data available on all three genes.

We expected to find more transcriptional enhancers of *ELF5* within the proteins we examined in this study. However, searching based on computer-based transcription factor motif prediction matrices is not a comprehensive method for identifying promoter regulators. Promoter regulation is dependent upon assembly of multi-protein complexes, some of which involve transcription factors that do not contact DNA in a conventional manner through their recognised DNA-binding domains interacting with a binding motif. Instead, factors contact DNA non-conventionally due to modification by protein interactions; for example, the Pax5-Ets-1 interaction, where Pax-5 recruits Ets-1 upon DNA binding. The interaction with Pax-5 increases Ets-1 DNA binding specificity to the sub-optimal EBS, GGAG (Garvie et al., 2001).

Secondly, transcription factors may be involved in the transcription complexes by protein partnerships only: that is, they bind a DNA-binding protein and connect it with other transcriptional proteins via a 'bridge' formation. These factors are therefore necessary for transactivation, but do not themselves contact the DNA. For example, p53 represses the TATA-less promoter of the *ets* family genes, *ETS-1* and *ETS-2*. It has been identified in protein complexes formed on oligonucleotides derived from TATA-less promoters but does not seem to directly contact DNA, at least through its traditional DNA-binding motif (Iotsova et al., 1996). Presumably, similar mechanisms are active in other *ets* promoter contexts, including the TATA-less *ELF5* promoter, particularly considering the recent and rapidly increasing number of discoveries of non-conventional, non-DNA contacting, promoter regulation (Chapter 1). While our study did not preclude identification of these interactions, it did focus on examining transcription factors with predicted optimal DNA binding motifs. This increases the likelihood that the regulators we examined would be those that bound directly to DNA in the traditional paradigm. Identification of factors that form protein:protein bridges, or non-conventional DNA:protein interactions, on the *ELF5* promoter will require advances in understanding the factors that do bind the DNA and then characterising those proteins they interact with when bound to the promoter, using ChIP and possibly Y-2-H analysis.

7.3 Alternative splice isoforms of ELF5 *are functional transcription factors*

ELF5 itself may be dependent upon interaction with other transcription factors to act on downstream target promoters. No transactivation domain (TAD) has been identified within the full-length, p30, isoform of ELF5. This is not unheard of within *ets* factors, though most possess TADs that do not display distinct similarities to each other. The *ets* family protein, Prf, binds the PU.1 EBS but does not appear to possess its own transactivation domain: it is possibly an antagonist for Spi-B and PU.1 binding, acting in a dominant-negative fashion. Alternatively, it may require interaction with protein partners to be transcriptionally active (Hashimoto et al., 1999). We presume that ELF5 has no TAD either, as there are no sequences that possess homology to a known TAD; indeed, there is little to the protein but the *pointed* and *ets* domains joined by a 45 amino acid central domain. N- and C- terminal tails are also minimal (45 and 12 amino acids respectively), but isoforms of the protein undeniably transactivate target promoters such as WAP (Thomas et al., 2000), PSA (Chapter 5; Oettgen et al., 1999b) and BRCA1 (Chapter 5).

The p20 isoform lacks the pointed protein-interaction domain and part of the 45 amino acid central domain. Both isoforms of ELF5 studied in our laboratory are, however, capable of transactivating, implying that transactivation or repression capability is provided by protein interaction partners within the transcriptional activation complex. Co-transfection of both p20 and p30 isoforms with the BRCA1α downstream target promoter did not alter the degree of transactivation provided by p20 (data not shown), indicating that the two isoforms are neither competing for binding sites, nor potentiating each others' activity.

However, both isoforms have not been detected simultaneously by RT-PCR in any organ or cell line, reiterating that they are required for different functions. While p20 lacks the canonical protein interaction domain, *ets* proteins are known to interact with partners through other domains including the *ets* DNA-binding domain. The elimination of the pointed domain sequence in p20 is functionally significant. p20 and p30 must interact with different proteins according to the difference in their sequence, as the outcome (binding to an EBS and action on the target gene promoter) is different. There is no significant homology between the N- and C-terminal tails or the central domain of ELF5 and those of other *ets* proteins, which if present could indicate a common protein partner between ELF5 and any other *ets* protein. Delineating which protein interactions are important for ELF5 activity, and distinguishing those that interact with either p20 or p30, will require techniques such as Y-2-H and co-IP. Finding these will contribute greatly to understanding mechanisms by which ELF5 is controlled, and also its downstream effects.

7.4 ELF5 plays different roles in hormone-dependent compared with non-hormonally regulated organs, and therefore in the carcinomas derived from these organs

Delineating the mechanisms that control *ELF5* activity in cellular growth, differentiation and maintenance of differentiation will also provide information on why the protein is relevant to carcinogenesis. Expression data (Chapter 3;(Zhou et al., 1998);Appendix VI) and cell culture studies (Oettgen et al., 1999b), demonstrate that *ELF5* is important for epithelial differentiation and possibly proliferation, depending upon the organ being examined. ELF5 is a potential regulator of genes that are markers of differentiation, as it

binds to EBS in the promoters of *SPRR2A*, expressed during cornification of the epidermis; the simple epithelium-specific keratin type II *EndoA*; and promoter of three genes expressed in the salivary glandular epithelium, *CRISP-1, CRISP-3* and *PSP* (Oettgen et al., 1999b).

ELF5 expression is altered in at least three carcinoma types, breast, prostate and kidney, compared to the normal tissues. This change in expression will dysregulate the tight control usually imposed over downstream target genes that include tumour suppressor genes such as *BRCA1* (Chapter 5) and tumour antigens such as *PSA* (Chapter 5; Oettgen et al., 1999b) and also of the differentiation genes mentioned previously. This may explain why alterations in ELF5 expression could assist in causing the propensity of cells to de-differentiate, leading to cancer.

In the breast and prostate, this study shows that human ELF5 protein is not lost in cancer tissue compared with normal tissue, and indeed may be over-expressed in the abnormal rather than normal epithelial cells. Contrary to predictions that the gene would undergo LOH at the 11p13 locus in solid tumours, the *ELF5* locus does not appear to be deleted in breast carcinoma DNA compared to normal DNA. The p20 isoform of *ELF5* can also regulate the crucial breast tumour suppressor *BRCA1 in vitro*, implying that lack of *ELF5* could play a part in downregulation of this transcript in sporadic breast carcinogenesis *in vivo*. These data imply that while *ELF5* plays a critical role in regulation of epithelial-specific genes and therefore in epithelial cell differentiation, it does not appear to function as a tumour suppressor and should be investigated further to determine whether it functions

as part of the *ets* oncogene cluster, rather than as a tumour suppressor, in hormonally-regulated organs. Due to lack of material, this study did not examine whether the *ELF5* locus carried any mutations, which could alter the functionality of the protein, in those cancers where the transcript was upregulated. If the transcript and protein levels are increased, but not functional, in these cases, *ELF5* may still be a tumour suppressor gene within the context of these two organs.

Although the kidney and prostate have the same origin early in development, their regulation and development diverge early in embryogenesis. Prostate development is regulated by androgens, unlike the kidney, which is not dependent upon steroid hormones. Breast development is also dependent upon secretion of the steroid hormones, estrogen and progesterone, and the peptide hormone, prolactin. ELF5 expression is downregulated in kidney cancer, though the protein is easily detectable in the normal organ. This situation contrasts with the detectability of the protein in normal (low to undetectable expression), compared with cancerous (strong expression), breast and prostate tissue. The expression status of the epithelium in these organs may reflect dual roles for ELF5 as a growth promoter or a growth suppressor, depending upon the organ context. This is not a phenomenon that has been observed among other *ets* transcription factors. Its signalling in prostate and breast is likely to be downstream of hormone induction (Zhou et al., 1998), although of course not in the non-hormone dependent kidney. Since *Elf5* is upregulated during pregnancy-associated mammary gland development, peaks in the proliferation phase and the heterozygous mice fail to develop large amounts of mammary epithelium, its role could be in assisting epithelial proliferation in hormone-dependent organs. Deletion of

ELF5 in mammary and possibly prostate carcinoma, therefore, may not be a selective advantage for the tumourigenic cells. In the kidney, ELF5 probably integrates different extra- or intra-cellular signals and acts in a different pathway. Unlike the breast, the adult kidney does not undergo phases of proliferation, differentiation and regression, and *ELF5* is expressed constantly in the epithelium. This could conceivably represent a role in maintenance of growth suppression, or terminal differentiation. Loss of *ELF5* expression in this context could result in a selective growth advantage for tumourigenic cells, explaining the significance of the difference between *ELF5* expression patterns in breast/prostate carcinoma and that derived from the kidney.

7.5 Conclusions

We have shown for the first time that the *ets* transcription factor, *ELF5*, is epithelial-specific, and is dysregulated in carcinomas of varied origin. *ELF5* is regulated transcriptionally by factors that have their own defined role in cancer, and it regulates cancer-associated targets genes differentially using at least two transcriptional isoforms. The gene behaves differently in carcinomas derived from hormonal-dependent compared with non-hormonally regulated organs, presumably reflecting its participation in different pathways in the development of the epithelium in these organs. *ELF5* appears to be a kidney-specific tumour suppressor gene, but to be differentially expressed in breast and prostate cancer, with its relevance to cancers of these organs yet to be determined. It acts as a tumour suppressor, potentially by regulating downstream target genes that include tumour suppressor genes. The mechanism by which its expression is downregulated in

kidney carcinoma has not yet been elucidated, though hypermethylation of the promoter, with associated silencing of expression, is a distinct possibility. Continuation of this study will address these questions, further elaborating on the details that define the role of *ELF5* as an epithelial-specific member of the *ets* oncogene/tumour suppressor gene family.

Appendix I. List of Oligonucleotides

Oligo-nucleotide Number	Oligonucleotide Name	Sequence (5′-3′)	T_m
***Elf5* null mouse genotyping**			
1	5′-ex-ko-B2	GCACACCCAGAATTGAAGATTCC	56
2	Neo-ko-B2	ATTCGCCAATGACAAGACGC	60
3	3′-in-ko	CCTTCACTGCACGTGGACTG	61
Oligonucleotides were used in a ratio of (1:1:2) respectively in a multiplex Taq PCR using Optiprime Buffer #5. 35 cycles of PCR were performed with an annealing temperature of 50°C and 1 minute extension.			
Chapter 3 **Production of Ese-3 recombinant protein**			
4	mESE3cdsBamHIF	CTGTTGGATCCCTTGCAGATCATG	59
5	mESE3SacIR-T	CAGTTCTCATGAGCTCATCCATCCA	59
Oligonucleotides 4 and 5 were used in 40 cycles of PCR using *Pfx* polymerase. 10 cycles of touchdown PCR were performed, with a starting annealing temperature of 55°C, decreasing 1°C per cycle until it reached 45°C. 30 cycles ensued using this annealing temperature. Extension was performed at 68°C for 1:20 minutes.			
Chapter 4 **h*ELF5* promoter characterisation**			
6	Prom-6F	CCAGCACTATTAGCTCCTCTTC	50
7	Prom-5F	TGGTCATGCAGTTGCATCAACT	55
8	Prom-4F	CCATGCACCAAGAATCATAGTAG	51
9	Prom-3F	ATGGGCTCCAGAGGCAGGCA	66
10	Prom-2F	AGATGGAGTCTCACTCTGTTGCC	54
11	Prom-1F	TTCAATCCCACTTCCTCCTT	58
12	Exon1-1R	GAGATGGACGAGCAGACCC	62
Oligonucleotides 6-11 were used as forward primers in a reaction with 12 as the reverse primer. *Pfx* polymerase (Invitrogen) was used in PCR with an annealing temperature of 52°C and 1-6 minutes extension at 68°C.			
11	Prom-1R	ACCTGTAGCCTTGTGATATAAGTGA	66
12	Exon1-1F	TGTCACACTGTATGTCACCGTC	62
13	Intron1-1F	GGATGAATCGTAAGGGGTGTC	64
14	Exon 2-1R	GGTAGATACCTCAGGACAACTGGATA TT	70
15	Intron1-1R	GCCAGTTTGCCAGAGGAGGA	64
All other promoter/intron PCRs were performed with an annealing temperature of 55°C and extension time of 2 minutes at 68°C, using the *Pfx* enzyme.			
Chapter 5 **Site-directed mutagenesis of the BRCA1α promoter**			
16	BRCA1HindIIIF	GCGATCTAAGTAAGCTTTATGGCAA	55
17	BRCA1ets#3mtR	CCGGATGACGTAAAATTAAAGAGAC	55
18	BRCA1ets#3mtF	GTCTCTTTAATTTTACGTCATCCGG	55
19	BRCA1HindIIIR	ACATGACCGGAATGCCAAGCTTCCA	63
20	BRCA1HindIIIets#4mtR	ATGCCAAGCTTCCATTAAGTCTCAGC	60

Primers 16+17, 18+19, 16+19 and 16+20 were used in PCR with *Pfu* polymerase (Promega). 40 cycles of PCR were performed with an annealing temperature of 55°C and 1 minute extension.			
EMSA Oligonucleotides			
21	BRCA1promets#3wtF	GTCTCTTTCCTTTAACGTCATCCGG	59
22	BRCA1promets#3wtR	CCGGATGACGTTAAAGGAAAGAGAC	59
23	BRCA1promets#3mtF	GTCTCTTTAATTTTACGTCATCCGG	55
24	BRCA1promets#3mtR	CCGGATGACGTAAAATTAAAGAGAC	55
25	BRCA1promets#4wtF	GCTGAGACTTCCTGGAAGCTTGGCAT	64
26	BRCA1promets#4wtR	ATGCCAAGCTTCCAGGAAGTCTCAGC	64
27	BRCA1promets#4mtF	GCTGAGACTTAATGGAAGCTTGGCAT	62
28	BRCA1prom1ets#4mtR	ATGCCAAGCTTCCATTAAGTCTCAGC	62
29	E74F	GATCATAACCGGAAGTAACT	56
30	E74R	GATCAGTTACTTCCGGTTAT	56
31	E74mtF	GATCATAACCATAAGTAACT	54
32	E74mtR	GATCAGTTACTTCTGGTTAT	54
Oligonucleotides 21-32 were not used in PCR but as double-stranded oligonucleotides for EMSA.			
Construction of PSA promoter-reporter construct			
33	PSAprom5′	ATTGGAATTCCACATTGTTTGCTGCA CG	66
34	PSAprom 3′	CTCTCCGGGTGCAGGTGGTAAGCTT	64
Elongase enzyme mix was used, with a final concentration of 1.6mM $MgCl_2$. 35 cycles of PCR were performed with an annealing temperature of 55°C and 1 minute extension at 68°C.			
Construction of 5′-end deletions of ELF5 for production of truncated proteins			
35	hELF5Δ33BamHIF	CGTAGGATCCGCCTTTGAGCATCAGA CAG	68
36	hELF5Δ33SacIR	CATGGAGCTCAGCTTGTCTTCCTGCC ACCC	72
Oligonucleotides 35 and 36 were used in PCR with *Pfx* polymerase. 30 cycles of PCR with an annealing temperature of 55°C and extension time of 1 min 20 sec at 68°C were performed.			
Chapter 6 **Analysis of GAAA repeat microsatellite marker**			
37	GAAA-1F	GTGACTGAGTGAGATTCTGT	58
38	GAAA-1R	AATGAGACTTTGTCACCAGC	58
Oligonucleotides 37 and 38 were used with *Pfx* polymerase. 35 cycles of PCR were performed with an annealing temperature of 53°C and an extension time of 1 minute.			
Construction and analysis of MCF7 Tet-On ELF5/EGFP expressing cell lines, 786-O ELF5 expressing cell lines and endogenous expression of mRNAs in cancer and normal tissue			
39	EGFP-N	CGTCGCCGTCCAGCTCGACCAG	68
40	EGFP-C	CATGGTCCTGCTGGAGTTCGTG	59
Oligonucleotides 39 and 40 were used in PCR with *Taq* polymerase. 35 cycles of PCR were performed with an annealing temperature of 55°C and an extension time of 1 minute.			
41	GAPDH5′	CTGCCACCCAGAACACTGT	52
42	GAPDH3′	GCTCATTTCCTGGTATGAC	52
Oligonucleotides 41 and 42 were used in PCR with *Taq* polymerase and 2mM $MgCl_2$. 35 cycles of PCR were performed with an annealing temperature of 55°C and an extension time of 40 sec.			
43	TET-ON-F	GGACGAGCTCCACTTAGACG	60
44	TET-ON-R	AGGGCATCGGTAAACATCTG	60

Oligonucleotides 43 and 44 were used in PCR with *Taq* polymerase and 2mM $MgCl_2$. 35 cycles of PCR were performed with an annealing temperature of 55°C and an extension time of 40 sec.			
45	CK8F	CTGGAGTCTCGCCTGGAAGG	66
46	CK8R	CCTCGTACTGTGCCTTGACC	64
Oligonucleotides 45 and 46 were used in PCR with *Taq* polymerase. 35 cycles of PCR were performed with an annealing temperature of 59°C and an extension time of 40 sec.			
47	BRCA12124F	GAAGTCAGAGGAGATGTG	54
48	BRCA12124R	CAGTAGTGGCTGTGGGGG	60
Oligonucleotides 47 and 48 were used in PCR with *Taq* polymerase. 35 cycles of PCR were performed with an annealing temperature of 55°C and an extension time of 1 min.			
49	hELF5-18	TGCCTTTGAGCATCAGACAG	60
50	hELF5-19	AGTATCATCTTGTTCGGAGG	58
Oligonucleotides 49 and 50 were used in PCR with *Taq* polymerase. 35 cycles of PCR were performed with an annealing temperature of 55°C and an extension time of 40 sec.			
Detection of *ELF5* in normal organ RNAs			
51	Ma-F	TGATTCCTGCTCTGGTGAAACA	55
52	Ma-R	ACATTTTCTCATCAATCACTGGTATGT	54
Oligonucleotides 51 and 52 were used in PCR with *Taq* polymerase. 35 cycles of PCR were performed with an annealing temperature of 55°C and an extension time of 40 sec.			
53	MaProbe	CAGTCCACCTAGCTGTCAGTCACTGA TA	59

Appendix II. List of Solutions

Unless otherwise specified, chemicals were obtained from BDH Biochemicals. Solutions were prepared using Millipore MilliQ H_2O (Millipore Corporation, USA).

General Solutions

10 X PBS (per litre)
8 g NaCl
200 mg KCl
1.44 g Na_2HPO_4
240 mg $KHPO_4$
adjust pH to 7.4

20 X SSC (per litre)
175.3 g NaCl
88.2 g sodium citrate
adjust pH to 7.0

50 X TAE (per litre)
242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA
adjust pH to 8.0

5 X TBE (per litre)
52g Tris base
27.5g boric acid
2 ml 0.5M EDTA (pH 8.0)

1 X TE
10 mM Tris-Cl (pH 8.0)
1 mM EDTA (pH 8.0)

6 X DNA loading dye
0.25 % (w/v) bromophenol blue
0.25 % (w/v) xylene cyanol FF
40 % (w/v) sucrose

Bacterial Work

LB liquid medium (per litre)
10 g bacto-tryptone
5 g bacto-yeast extract
10 g NaCl
adjust pH to 7.0

LB plates
LB + 15 g/litre agar

Glycerol stocks of bacteria
Add 0.2 volumes of 100% glycerol to the culture medium; store at -80°C

SOC liquid medium (per litre)
10 g bacto-tryptone
2.5 g bacto-yeast extract
250 mg NaCl
2.5 mM KCl
adjust pH to 7.0
5 ml 2 M $MgCl_2$
10 ml 2 M glucose

Antibiotics
Ampicillin sodium salt 50 µg/ml
Kanamycin sulphate 12.5 µg/ml
Chloramphenicol 37µg/ml in ethanol

DNA Work

DNA Extraction from Bacteria

Solution I
50 mM glucose
10 mM EDTA (pH 8.0)
25 mM Tris-Cl (pH 8.0)

Solution II
0.2 M NaOH
1 % (w/v) SDS (Roche Diagnostics)

Solution III
5 M KOAc
11.5 % (v/v) glacial acetic acid

DNA Extraction for Mouse Genotyping

Ear clip buffer
10 mM Tris-Cl (pH 8.3)
50 mM NaCl
0.2 % (w/v) Tween 20
10 μg/ml proteinase K

DNA Extraction from Mammalian Cells

Cell lysis buffer
0.1 M Tris-Cl (pH 7.5)
5 mM EDTA (pH 8.0)
0.2 % (w/v) SDS
0.2 M NaCl
mg/ml proteinase K

RNase A
10 mg/ml RNase A
10 mM Tris-HCl (pH 7.5)
15 mM NaCl

Phenol-Chloroform
50ml saturated phenol (pH 8.0) (Sigma)
48 ml chloroform
2ml isoamyl alcohol

DNA extraction from PBLs

Blood lysing solution
10 mM Tris (pH 8.0)
10 mM NaCl
10 mM EDTA

5 X ANE
0.05 M sodium acetate
0.5 M NaCl
5 mM EDTA (pH 8.0)
2.5 % (w/v) SDS

Hybridisation Solutions

Hybridisation solution
10 % (w/v) dextran sulphate (Clontech)
1 % (w/v) SDS
1 M NaCl
100 μg/ml denatured herring sperm DNA

Oligonucleotide hybridisation solution
5 X SSC
5 X Denhardt's
1 % (w/v) SDS
100 μg/ml denatured herring sperm DNA

100 x Denhardt's solution
2 % (w/v) Bovine Serum Albumin Fraction V (BSA)
2 % (w/v) Ficoll
2 % (w/v) Polyvinylpyrrolidone (PVP)

Microsatellite Analysis

6 % urea-polyacrylamide gel (300ml)
60 ml (19:1 Acrylamide/N,N'-methylene-bis-acrylamide) (BioRad)
30 ml 5 X TBE
7M urea

5 X formamide loading dye
95 % (v/v) deionised formamide
10 mM EDTA (pH 8.0)
0.1 % (w/v) bromophenol blue
0.1 % (w/v) xylene cyanol FF

RNA Work

Total RNA Extraction

Solution D
250 g Guanidinium Thiocyanate
17.6 ml of 0.75 M Sodium Citrate (pH 7.0)
26.4 ml of 10 % (w/v) Sarkosyl
293 ml H_2O

PolyA$^+$ RNA Extraction

Extraction buffer
0.5 % (w/v) SDS
0.1 M NaCl
20 mM Tris (pH 7.5)
1 mM EDTA (pH 8.0)

Binding buffer
0.1 % (w/v) SDS
0.5 M NaCl
10 mM Tris-Cl (pH 7.5)
1 mM EDTA (pH 8.0)

Wash buffer
0.1 % (w/v) SDS
0.15 M NaCl
10 mM Tris-Cl (pH 7.5)
1 mM EDTA (pH 8.0)

Elution buffer
0.1 % (w/v) SDS
10 mM Tris-Cl (pH 7.5)
1 mM EDTA (pH 8.0)

Protein Work

Protein extraction

RIPA buffer (pH 7.6)
10 mM Tris
150 mM NaCl
0.1 % (w/v) SDS
1 % (w/v) Na deoxycholate
1 % (v/v) Triton X 100

Recombinant protein preparation

Guanidine lysis buffer
6 M guanidine-HCl
20 mM Tris-Cl (pH 8.0)
50 mM NaCl

Renaturation buffer
20 mM Tris-Cl (pH 8.0)
50 mM NaCl
0.1 % (v/v) NP-40
0.1 % (v/v) Tween-20
0.1 % (v/v) Triton X 100

Native protein elution buffer
20 mM Tris-Cl (pH 8.0)
100 mM NaCl
100 mM Imidazole

Western Blots

2 X EDTA sample buffer
125 mM Tris-Cl (pH 6.8)
2 % (w/v) SDS
20 % (v/v) Glycerol
1.4 M β-mercaptoethanol
2 % (w/v) Bromophenol blue
2 mM EDTA (pH 8.0)

SDS-PAGE gel - separation layer
12 % (v/v) acrylamide (29:1 acrylamide/N,N'-methylene-bis-acrylamide) (BioRad)
0.1 % (w/v) ammonium persulphate
0.1 % (v/v) TEMED (tetramethylethylenediamine; Sigma)
0.1 % (w/v) SDS
375 mM Tris-Cl (pH 8.8)

SDS-PAGE gel - stacking layer
5 % (v/v) acrylamide (29:1 acrylamide/N,N'-methylene-bis-acrylamide)
0.1 % (w/v) ammonium persulphate
0.1 % (v/v) TEMED
0.1 % (w/v) SDS
62 mM Tris-Cl (pH 6.8)

SDS-PAGE running buffer
25 mM Tris-HCl
192 mM glycine
0.1 % (w/v) SDS

Semi-dry Transfer

Anode Buffer I
0.3 M Tris
10 % (v/v) methanol
Adjust pH to 10.4

Anode Buffer II
25 mM Tris
10 % (v/v) methanol
Adjust pH to 10.4

Cathode Buffer
25 mM Tris
40 mM glycine
10% (v/v) methanol
Adjust pH to 9.4

10 X TBST
0.2 M Tris-Cl (pH 8.0)
1.4 M NaCl
1 % (v/v) Tween 20

Coommassie Brilliant Blue staining solution
0.25 % (w/v) Coommassie Brilliant Blue
R-250
50 % (v/v) methanol
10 % (v/v) glacial acetic acid

Coommassie Brilliant Blue destaining solution
10 % (v/v) methanol
7.5 % (v/v) glacial acetic acid

DNA-Protein Interaction Work

EMSA

6 % polyacrylamide gel (300ml)
60 ml (19:1 Acrylamide/N,N'-methylene-bis-acrylamide) (BioRad)
30 ml 5 X TBE
210 ml dH_2O

5 X EMSA Binding Buffer

50 mM HEPES (pH 7.8)
10 mM $MgCl_2$
200 mM KCl
5 mM DTT
25% glycerol
0.25 μg/ml polydI.dC-dI.dC

Mammalian Cell Culture and Transfections

Cell freezing media
95% (v/v) FCS
5% (v/v) DMSO

Normalisation of luciferase assays

β-galactosidase sample buffer
700 µl β-mercaptoethanol
200 µl 1 M $MgCl_2$
1.56 g $Na_2HPO_4.2H_2O$
1.41 g $NaH_2PO_4.2H_2O$
0.133g2-nitrophenyl-β-D-galactopyraniside
MQ H_2O to 100 ml

Histology and Immunohistochemistry

Neutral buffered formalin
10% formaldehyde in PBS

Paraformaldehyde
4% (w/v) paraformaldehyde in PBS

Acid ethanol
0.5% (v/v) hydrochloric acid
70% (v/v) ethanol

Appendix III. List of Suppliers

Amersham Pharmacia Biotech.	Buckinghamshire, UK
Amrad	Kew, VIC, Australia
Bartelt Instruments	Heidelberg West, VIC, Australia
BDH Biochemicals / Merck	Poole, UK
Beckman Instruments	Fullerton, CA, USA
Becton Dickinson (BioSciences)	Lincoln Park, NJ, USA
BioRad Laboratories, Inc.	Hercules, CA, USA
DAKO	Carpinteria, CA, USA
Difco	Detroit, MI, USA
Fluka	Buchs SG, Switzerland
Fronine Laboratory Supplies	Riverstone, NSW, Australia
Gibco-BRL (Life Technologies)	Paisley, UK
Hoefer	San Francisco, CA, USA
Kodak Scientific	Rochester, NY, USA
Lab Aids	Sydney, NSW, Australia
Leica Instruments	Nussloch, Germany
Millipore Corporation	Bedford, MA, USA
New Brunswick Scientific	Edison, NJ, USA
New England Biolabs	Beverly, MA, USA
Novagen Inc.	Milwaukee, WI, USA
Nunc	Naperville, IL, USA
PharMingen	La Jolla, California, USA
Pierce	Rockford, IL, USA
Progen Industries	Rockford, IL, USA
Proligo	Boulder, CO, USA
Promega	Madison, WI, USA
Qiagen	Chatsworth, CA, USA
Roche Molecular Biochemicals	Mannheim, Germany
Sartorius	Goettingen, Germany
Scientifix	Cheltenham VIC, Australia
Sigma Chemical Company	Saint Louis, MO, USA
Stratagene	La Jolla, CA, USA
SuperBioChips Laboratories	Seoul, South Korea
Zymed	South San Francisco, CA, US

Appendix IV. List of Equipment

Agarose gel electrophoresis	Mini-sub Cell GT, Bio-Rad
β-scintillation counter	LKB, 1217 RACKBETA, Wallace Scintillation Counter
Centrifuges (large)	J2-21 M/E, Beckman Biofuge stratos, Heraeus Instruments
Cell incubator	HERA Cell, Heraeus Instruments
DNA sequencer	Model 373A, Applied Biosystems Automatic Sequi-Gen I, Bio-Rad (microsatellite analysis)
Electroporation apparatus	Gene Pulser, Bio-Rad
Homogeniser	Ika Ultra Turrax T25, Crown Scientific
Hybridisation ovens	XTRON H2002, Bartelt Instruments
Paraffin embedding centre	EG1160, Leica Instruments
Paraffin microtome	RM2135, Leica Instruments
Paraffin processor	TP1020, Leica Instruments
Protein gel caster	Mighty Small multiple gel caster SE200, Hoefer Scientific Instruments, Amersham Pharmacia
Microcentrifuge	1-15 Sigma, Quantum Scientific
PCR machines	GeneAmp PCR System 2400 and 9600, Applied Biosystems
SDS-PAGE electrophoresis apparatus	MightySmall II SE250, Hoefer Scientific Instruments, Amersham Pharmacia
Shaking incubator	Innova 4300, New Brunswick Scientific
Spectrophotometer	Lambda Bio 20, Perkin Elmer
UV illuminator	Gel Doc 1000, Bio-Rad
Western transfer apparatus	TE77 SemiPhor, Hoefer Scientific Instruments, Amersham Pharmacia

Appendix V. Output from transcription factor binding site searches

NB. Position number 1 on the transcription factor site search results corresponds to position –9058 of the h*ELF5* sequence relative to the p30 translational start codon (Chapter 4).

*V.I BCM Search Launcher search for predicted transcription factor binding sites in the h*ELF5 *promoter*

```
00001  ATTTCAATCC CACTTCCTCC TTTTGCCACT GGGGAAAAAT GAAGCCCAGA  00050
               = =========(13.6900) [redacted]
                 =========(12.7420) [redacted]
00051  GAAGTCCAGG TTACCCAGCC AAGATAGGGG TCTAGGCAGT CACCTATCTT  00100
                      ---- -------(12.4886) Evi-1 M00082
                       === ========== =(13.3530) GATA-1,GATA-1A M00126
00101  CCTTCAACAA TTTTTCCATG ATGACACCCC GAATCCTCTC ACGAACTGCC  00150
00151  TTTTGTTTCT GCAGACTATA TGCGTATTTG TATTTGTGTG TTTGTAACCA  00200
                            --------- ----(13.6373) Freac-6 M00289
00201  TGCCCGGTGA TCCCAAGAAA ACATAACAAA CCAGTCCTTG CTTTCGCTTT  00250
00251  AAAGCTTGGA GTCTGCCATT TGAATACAAC ATCTCGGCTG CCCAAGATGG  00300
          ------ ----------(13.1467) E47,Th1 M00222
00301  CTAGAAGCAG AATGCAAAAA GGCACAGGGT TATAAATACC TGTCTCATAG  00350
               == ========(13.2747) abaA Q00209
00351  ATGACCTGGG ACACTGTGCT TTGCAGCCTA AATTAGGCAG AGTTTCTGTT  00400
        ========= =(13.2678) [redacted]
00401  GTCACGAAGA AGTACTAAAA AGCGGGCAGT TCTCAGCGAG ACACCTGAGA  00450
00451  GCTGGCATCC ACATGAGGAG AGGCCCCATC ACTTACATTA CACTCATGAA  00500
00501  GCCCAGAGAT GTTAGCTACA TTTTTCTAGG TAGCACAGCT AGGAAATGAT  00550
00551  GGACACTGAA TTTGAATCTA AAGGCAACTA GCTGCAAAAC CTGAGGTCTT  00600
00601  AACACTGGGC CTATAATGCT CTTCCTTCTC ACCACATGGG AAAAACGGAG  00650
                                       -  ---------- -(12.2859) Max1,
                                                          c-Myc M00123
                                                 ==== =======(13.5624)
                                                                  D1 M00120
00651  AAAGACATGA TCTTACAAAA GCACTGGGCT CAGCCAGCCT GGGAGAGAGG  00700
00701  CAGCGCAGAC CGGCACCAGG GTGGAATCTC TGTGTTTCCC CTCCAGCTTG  00750
00751  CACAGAGGAA AATTCCTCAC CAAACGCACG ATCCCCGCAC AGCTCCCAGG  00800
        ======== ====(14.4539) V$NFAT_Q6 M00302
00801  GACAAGAATT TTCTGCTTGT TTACTGAGTC CCCTGGGCTG GGAGTGGGGG  00850
                  ------- ---------(16.6263) Freac-3 M00291
                  ------- -------(13.4638) HNF-3beta M00269
                   ------ -------(12.1492) Freac-6 M00289
                          --------- --(12.8358) NF-E2,NF-E2_p45 M00037
00851  GTTGCAGGGC AGGGGTGAGC TGCGCACAAA AGCAGGATAA AGGTAAACTT  00900
                                    ========(12.3687) LVb-binding
                                                      factor Q00102
00901  TCTGCATATG AGAACCATTT CCCCCCCTCC AAGGAGCCGT GTCACACTGT  00950
                         -- ---------- -(12.4508) MZF-1 M00084
00951  ATGTCACCGT CATCAAAGGG GCTGTGCGTA AACCTGAAAA ACCAAACGGA  01000
```

```
01001  CCTGTCTGTA GGTGTCACTT ATATCACAAG GCTACAGGTG TCTTTATTTC  01050
01051  CACTGCACGC TGGTGCTGGG AGCGCCTGCC TTCTCTTGCC TTGAAAGCCT  01100
01101  CCTCTTTGGA CCTAGCCACC GCTGCCCTCA CGTAAGCGCC TTTTCTTTGC  01150

01151  TTTTTTGGAT GAATCGTAAG GGGTGTCAGG GCTCAGAGGC TCGGCCTGGG  01200
                                                 ========···(14.8138)

01201  GCCTGGGTCT GCTCGTCCAT CTC  01223
    ···===(14.8138) AP-2 Q00008
```

*V.II MatInspector search for predicted transcription factor binding sites in the h*ELF5 *promoter*

```
Parameters:
~~~~~~~~~~~~~~~~~~~~
sequence file:   ELF5promoter.seq
core sim:        0.75
matrix sim:      0.85

Explanation for column output:
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
        Matrix positions correspond to sense strand numbering, but all
        sequences are given in 5'-3' direction.

        n/a in column 'core simil.' indicates, that no core search
        was conducted.

        Capital letters within the sequence indicate the core string.
```

Matrix Name	Position(str) of Matrix	Core Simil.	Matrix Simil.	Sequence
Inspecting sequence ELF5 promoter (1 - 1223):				
Transcription factor binding sites of interest are highlighted in blue.				
V$IK2_01	4 (-)	1.000	0.952	aagtGGGAttga
V$GKLF_01	9 (-)	0.873	0.865	aaggaggaagTGGG
V$GKLF_01	10 (-)	0.768	0.884	aaaggaggaaGTGG
V$NKX25_01	11 (-)	1.000	0.884	ggAAGTg
V$BARBIE_01	13 (-)	1.000	0.879	ggcaAAAGgaggaag
V$USF_Q6	25 (+)	0.864	0.880	gcCACTgggg
V$CEBPB_01	27 (+)	0.986	0.892	cactgggGAAAaat
V$MZF1_01	28 (+)	1.000	0.972	actGGGGa
V$IK2_01	28 (+)	1.000	0.922	actgGGGAaaaa
V$IK1_01	28 (+)	1.000	0.887	actgGGGAaaaat
V$NFAT_Q6	29 (+)	1.000	0.958	ctgggGAAAaat
V$TCF11_01	31 (-)	0.782	0.872	TTCAtttttcccc
V$NF1_Q6	57 (-)	1.000	0.940	tctTGGCtgggtaacctg
V$RFX1_01	58 (-)	0.945	0.898	tcttggctggGTAAcct
V$GKLF_01	67 (+)	0.951	0.857	agccaagataGGGG
V$GATA1_03	68 (+)	1.000	0.931	gccaaGATAggggt
V$GATA1_02	68 (+)	1.000	0.991	gccaaGATAggggt
V$GATA1_04	69 (+)	1.000	0.956	ccaaGATAggggt
V$LMO2COM_02	71 (+)	1.000	0.991	aaGATAggg
V$GATA_C	72 (+)	0.891	0.935	aGATAGgggtc
V$AP1FJ_Q2	84 (-)	1.000	0.966	ggTGACtgcct
[redacted]	84 (-)	1.000	0.951	ggTGACtgcct
	84 (-)	1.000	0.934	ggTGACtgcct
V$DELTAEF1_01	88 (+)	1.000	0.949	agtcACCTatc
V$GATA_C	89 (-)	0.891	0.935	aGATAGgtgac
V$TCF11_01	89 (+)	1.000	0.894	GTCAcctatcttc
V$GATA1_02	90 (-)	1.000	0.958	aggaaGATAggtga
V$GATA1_03	90 (-)	1.000	0.948	aggaaGATAggtga

V$GATA1_04	90	(-)	1.000	0.953	ggaaGATAggtga
V$LMO2COM_02	92	(-)	1.000	0.943	aaGATAggt
V$CEBPB_01	96	(-)	0.873	0.874	tgttgaaGGAAgat
[redacted]	99	(+)	1.000	0.873	TTCCttcaa
V$SRY_02	103	(+)	1.000	0.911	ttcaACAAtttt
V$SOX5_01	104	(+)	1.000	0.985	tcaaCAATtt
V$NFAT_Q6	110	(-)	1.000	0.972	tcatgGAAAaat
V$TCF11_01	110	(-)	0.807	0.866	ATCAtggaaaaat
V$TCF11_01	113	(-)	1.000	0.992	GTCAtcatggaaa
V$AP1_Q2	120	(+)	1.000	0.889	gaTGACacccc
V$AP1FJ_Q2	120	(+)	1.000	0.918	gaTGACacccc
V$AP1_Q4	120	(+)	1.000	0.851	gaTGACacccc
V$CMYB_01	139	(-)	0.797	0.864	acaaaaggcaGTTCgtga
V$VMYB_01	141	(+)	0.876	0.858	acgAACTgcc
V$GKLF_01	149	(-)	0.937	0.875	gcagaaacaaAAGG
V$SRY_02	149	(-)	1.000	0.948	agaaACAAaagg
V$HFH8_01	151	(+)	1.000	0.876	tttTGTTtctgca
V$CEBPB_01	153	(-)	0.986	0.870	gtctgcaGAAAcaa
V$HNF3B_01	170	(+)	1.000	0.870	atgcgTATTtgtatt
V$HFH3_01	172	(+)	0.955	0.945	gcgTATTtgtatt
[redacted]	173	(-)	1.000	0.858	acaaatacAAATacg
[redacted]	173	(+)	0.889	0.876	cgtatttgtATTTg
V$SRY_02	174	(-)	1.000	0.857	aaatACAAatac
V$HNF3B_01	176	(+)	1.000	0.854	atttgTATTtgtgtg
V$HFH3_01	178	(+)	0.955	0.927	ttgTATTtgtgtg
V$HFH3_01	186	(+)	1.000	0.890	gtgTGTTtgtaac
V$HFH8_01	186	(+)	1.000	0.875	gtgTGTTtgtaac
V$E2_Q6	195	(+)	1.000	0.947	taaccatgccCGGTga
V$E2_01	195	(+)	1.000	0.940	taaccatgccCGGTga
V$E2_Q6	195	(-)	0.833	0.873	tcaccgggcaTGGTta
V$E2_01	195	(-)	0.852	0.895	tcaccgggcaTGGTta
V$USF_Q6	202	(-)	0.818	0.877	atCACCgggc
V$IK2_01	207	(-)	1.000	0.965	tcttGGGAtcac
V$NFAT_Q6	212	(+)	1.000	0.855	cccaaGAAAaca
V$HFH3_01	214	(-)	1.000	0.864	ttaTGTTttcttg
V$HNF3B_01	214	(-)	1.000	0.924	tgttaTGTTttcttg
V$HFH2_01	215	(-)	1.000	0.896	ttaTGTTttctt
V$HFH2_01	220	(-)	1.000	0.850	gttTGTTatgtt
V$SRY_02	222	(+)	1.000	0.913	cataACAAacca
[redacted]	226	(+)	0.847	0.855	acaaaCCAGtcc
V$GKLF_01	236	(-)	0.937	0.853	aagcgaaagcAAGG
V$CP2_01	237	(-)	0.909	0.895	gcgaaagCAAG
V$IRF2_01	240	(-)	1.000	0.885	ttaaagcGAAAgc
V$IRF1_01	240	(-)	1.000	0.909	ttaaagcGAAAgc
V$BARBIE_01	247	(+)	1.000	0.887	ctttAAAGcttggag
V$NF1_Q6	254	(-)	1.000	0.861	aaaTGGCagactccaagc
V$TH1E47_01	255	(+)	0.800	0.885	cttggagtCTGCcatt
V$AP4_Q5	283	(-)	1.000	0.897	ggCAGCcgag
V$NF1_Q6	295	(+)	1.000	0.878	agaTGGCtagaagcagaa
[redacted]	308	(+)	0.800	0.870	cagaatgcAAAAagg
[redacted]	309	(-)	0.889	0.861	ccttttgcATTCt
V$GKLF_01	315	(+)	0.789	0.869	caaaaaggcaCAGG
V$GKLF_01	316	(+)	1.000	0.865	aaaaaggcacAGGG
V$USF_Q6	321	(+)	0.864	0.861	ggCACAgggt
V$FREAC7_01	327	(+)	1.000	0.897	gggttaTAAAtacctg
V$HFH3_01	329	(-)	0.955	0.865	aggTATTtataac
V$HFH8_01	329	(-)	0.816	0.862	aggTATTtataac

V$XFD2_01	329 (+)	1.000	0.867	gttaTAAAtacctg
[redacted]	330 (+)	1.000	0.943	ttaTAAAtacctgtc
V$DELTAEF1_01	334 (+)	1.000	0.854	aaatACCTgtc
V$TCF11_01	343 (-)	1.000	0.988	GTCAtctatgaga
V$CDP_02	344 (+)	0.806	0.852	ctcATAGatgacctg
V$TCF11_01	344 (+)	0.798	0.867	CTCAtagatgacc
V$CDPCR3HD_01	346 (+)	0.930	0.939	cataGATGac
[redacted]	350 (+)	1.000	0.873	gaTGACctggg
[redacted]	350 (+)	1.000	0.888	gaTGACctggg
[redacted]	350 (+)	1.000	0.854	gatgACCTggg
V$RORA1_01	351 (-)	1.000	0.911	tgtcccaGGTCat
V$IK2_01	354 (+)	1.000	0.919	acctGGGAcact
V$BARBIE_01	362 (-)	1.000	0.854	ctgcAAAGcacagtg
V$PADS_C	365 (+)	0.865	0.856	tGTGCTttg
V$S8_01	374 (+)	1.000	0.943	cagcctaaATTAggca
V$NKX25_02	380 (-)	1.000	0.896	ccTAATtt
V$CMYB_01	388 (+)	1.000	0.891	cagagtttctGTTGtcac
V$RFX1_02	390 (-)	0.882	0.915	tcgtgacaacaGAAActc
V$ISRE_01	390 (+)	1.000	0.874	gaGTTTctgttgtca
V$VMYB_01	394 (-)	0.820	0.877	gacAACAgaa
V$VMYB_02	395 (-)	0.784	0.859	gacAACAga
V$AP1_Q2	396 (-)	1.000	0.859	cgTGACaacag
V$AP1FJ_Q2	396 (-)	1.000	0.913	cgTGACaacag
V$TCF11_01	401 (+)	1.000	0.886	GTCAcgaagaagt
V$GKLF_01	413 (+)	0.852	0.856	tactaaaaagCGGG
V$BARBIE_01	415 (+)	1.000	0.903	ctaaAAAGcgggcag
V$CMYB_01	419 (+)	0.797	0.851	aaagcgggcaGTTCtcag
V$VMYB_01	425 (-)	0.876	0.856	gagAACTgcc
V$E47_02	437 (-)	1.000	0.895	gctctCAGGtgtctcg
V$E47_01	437 (-)	0.786	0.874	ctcTCAGgtgtctcg
[redacted]	439 (+)	1.000	0.968	agacACCTgag
V$MYOD_01	439 (-)	1.000	0.898	tctCAGGtgtct
V$LMO2COM_01	439 (-)	1.000	0.947	tctCAGGtgtct
[redacted]	440 (+)	0.818	0.866	gaCACCtgag
V$MYOD_Q6	440 (+)	1.000	0.907	gaCACCtgag
[redacted]	441 (+)	0.856	0.921	aCACCTga
V$AP4_Q5	447 (-)	1.000	0.896	gcCAGCtctc
V$TCF11_01	455 (-)	0.798	0.853	CTCAtgtggatgc
V$SRF_Q6	457 (+)	0.861	0.879	atCCACatgaggag
V$LMO2COM_01	457 (-)	0.822	0.898	cctCATGtggat
V$MYCMAX_02	457 (+)	0.895	0.857	atcCACAtgagg
[redacted]	458 (+)	0.864	0.860	tcCACAtgag
[redacted]	459 (+)	0.876	0.936	cCACATga
[redacted]	459 (-)	0.817	0.854	tCATGTgg
V$CEBP_C	477 (-)	0.957	0.875	agtgtaatGTAAGtgatg
V$NKX25_01	480 (-)	1.000	0.930	gtAAGTg
V$CEBPB_01	480 (-)	0.930	0.889	gtgtaatGTAAgtg
V$E4BP4_01	481 (-)	1.000	0.875	tgtaatGTAAgt
V$VBP_01	482 (+)	1.000	0.904	cTTACattac
[redacted]	484 (-)	1.000	0.867	atgagtgtaATGTa
V$TCF11_01	493 (+)	0.798	0.870	CTCAtgaagccca
V$GATA2_03	505 (+)	0.794	0.860	agaGATGtta
V$GATA3_03	505 (+)	1.000	0.948	agAGATgtta
V$NFAT_Q6	520 (-)	1.000	0.854	acctaGAAAaat
[redacted]	525 (-)	1.000	0.856	tgctACCTaga
V$AP4_Q6	534 (+)	1.000	0.899	caCAGCtagg
V$AP4_Q5	534 (+)	1.000	0.900	caCAGCtagg

V$NFAT_Q6	538	(+)	1.000	0.928	gctagGAAAtga
V$TCF11_01	538	(-)	0.807	0.857	ATCAtttcctagc
V$TST1_01	555	(+)	1.000	0.878	actgAATTtgaatct
V$GFI1_01	557	(+)	1.000	0.859	tgaatttgAATCtaaaggcaacta
V$AP4_Q5	577	(-)	1.000	0.879	tgCAGCtagt
V$AP4_Q6	577	(-)	1.000	0.851	tgCAGCtagt
[redacted]	585	(+)	1.000	0.857	caaaACCTgag
	601	(+)	0.864	0.867	aaCACTgggc
V$MYCMAX_02	630	(+)	0.895	0.937	cacCACAtggga
V$LMO2COM_01	630	(-)	0.822	0.902	tccCATGtggtg
V$MYOD_01	630	(-)	0.833	0.864	tccCATGtggtg
[redacted]	631	(+)	0.864	0.868	acCACAtggg
	632	(-)	0.817	0.857	cCATGTgg
	632	(+)	0.876	0.933	cCACATgg
V$IK1_01	634	(+)	1.000	0.935	acatGGGAaaaac
V$IK2_01	634	(+)	1.000	0.955	acatGGGAaaaa
V$NFAT_Q6	635	(+)	1.000	0.970	catggGAAAaac
V$VMYB_01	641	(+)	1.000	0.944	aaaAACGgag
V$VMYB_02	641	(+)	1.000	0.913	aaaAACGga
V$TCF11_01	649	(-)	0.807	0.862	ATCAtgtctttct
V$GATA3_02	656	(-)	0.831	0.851	taaGATCatg
V$GATA3_03	656	(-)	1.000	0.850	taAGATcatg
V$GATA3_03	656	(+)	0.875	0.908	caTGATctta
V$CEBPB_01	660	(-)	0.930	0.927	tgcttttGTAAgat
V$SRY_02	661	(+)	1.000	0.869	tcttACAAaagc
V$VBP_01	662	(+)	1.000	0.856	cTTACaaaag
V$BARBIE_01	664	(+)	1.000	0.871	tacaAAAGcactggg
V$PADS_C	667	(-)	0.865	0.855	aGTGCTttt
V$USF_Q6	670	(+)	0.864	0.854	agCACTgggc
V$IK2_01	687	(+)	1.000	0.923	gcctGGGAgaga
V$LYF1_01	688	(+)	1.000	0.864	cctGGGAga
V$AP4_Q5	699	(+)	1.000	0.859	ggCAGCgcag
V$GFI1_01	717	(+)	1.000	0.916	cagggtggAATCtctgtgtttccc
V$GATA3_03	722	(-)	1.000	0.855	agAGATtcca
V$IK1_01	732	(-)	1.000	0.856	ggagGGGAaacac
V$NFAT_Q6	732	(-)	1.000	0.932	gagggGAAAcac
V$IK2_01	733	(-)	1.000	0.907	ggagGGGAaaca
V$MZF1_01	737	(-)	1.000	0.982	ggaGGGGa
V$NFAT_Q6	753	(+)	1.000	1.000	cagagGAAAatt
V$CREL_01	757	(-)	1.000	0.862	ggaattTTCC
V$MZF1_01	764	(-)	0.779	0.854	ggtGAGGa
V$IK2_01	778	(-)	1.000	0.881	tgcgGGGAtcgt
V$MZF1_01	782	(-)	1.000	0.975	tgcGGGGa
V$AP4_Q5	788	(+)	1.000	0.918	caCAGCtccc
V$AP4_Q6	788	(+)	1.000	0.895	caCAGCtccc
V$IK2_01	790	(-)	1.000	0.910	ccctGGGAgctg
V$IK2_01	795	(+)	1.000	0.892	cccaGGGAcaag
V$SRY_02	813	(-)	1.000	0.897	gtaaACAAgcag
V$HNF3B_01	813	(+)	1.000	0.877	ctgctTGTTtactga
V$FREAC2_01	814	(-)	1.000	0.939	actcagTAAAcaagca
V$XFD3_01	814	(-)	1.000	0.958	tcagtaAACAagca
V$FREAC3_01	814	(-)	1.000	0.916	actcaGTAAacaagca
V$FREAC4_01	814	(-)	1.000	0.851	actcagtaAACAagca
V$XFD2_01	814	(-)	1.000	0.882	tcagTAAAcaagca
V$HFH3_01	815	(+)	1.000	0.876	gcttGTTTactga
V$HFH8_01	815	(+)	1.000	0.864	gcttGTTTactga
V$NFE2_01	822	(+)	1.000	0.862	taCTGAgtccc

V$IK2_01	825 (-)	1.000	0.880	ccagGGGActca
V$AP2_Q6	828 (+)	0.976	0.857	gtCCCCtgggct
V$MZF1_01	829 (-)	1.000	0.948	ccaGGGGa
V$IK2_01	836 (+)	1.000	0.922	ggctGGGAgtgg
V$CMYB_01	841 (+)	1.000	0.863	ggagtgggggGTTGcagg
V$MZF1_01	843 (+)	1.000	0.858	agtGGGGg
V$CHOP_01	847 (-)	1.000	0.882	cccTGCAaccccc
V$RFX1_01	849 (-)	1.000	0.866	ccccctgcccctGCAAccc
V$AP4_Q5	865 (-)	1.000	0.918	cgCAGCtcac
V$AP4_Q6	865 (-)	1.000	0.869	cgCAGCtcac
V$BARBIE_01	875 (+)	1.000	0.863	cacaAAAGcaggata
V$GATA1_03	881 (+)	1.000	0.970	agcagGATAaaggt
V$GATA1_02	881 (+)	1.000	0.931	agcagGATAaaggt
V$GATA1_04	882 (+)	1.000	0.927	gcagGATAaaggt
V$CETS1P54_01	882 (+)	0.926	0.866	gcAGGAtaaa
V$GATA1_05	883 (+)	1.000	0.892	cagGATAaag
V$GATA3_02	883 (+)	1.000	0.870	cagGATAaag
V$GATA2_02	883 (+)	1.000	0.900	cagGATAaag
V$GATA1_06	883 (+)	1.000	0.888	cagGATAaag
V$LMO2COM_02	884 (+)	1.000	0.925	agGATAaag
V$GATA_C	885 (+)	1.000	0.935	gGATAAaggta
V$DELTAEF1_01	888 (-)	1.000	0.870	gtttACCTtta
V$CEBPB_01	896 (-)	0.986	0.912	atatgcaGAAAgtt
V$OCT1_02	899 (-)	1.000	0.912	tctcatATGCagaaa
[redacted]	915 (-)	1.000	0.905	gggggGAAAtgg
V$IK2_01	916 (-)	1.000	0.903	ggggGGGAaatg
V$MZF1_01	920 (-)	1.000	0.986	gggGGGGa
[redacted]	936 (-)	1.000	0.908	tgTGACacggc
	936 (-)	1.000	0.860	tgTGACacggc
V$TCF11_01	941 (+)	1.000	0.899	GTCAcactgtatg
V$AP1_Q2	948 (-)	1.000	0.934	ggTGACataca
V$AP1FJ_Q2	948 (-)	1.000	0.962	ggTGACataca
V$AP1_Q4	948 (-)	1.000	0.908	ggTGACataca
V$ATF_01	952 (-)	1.000	0.884	tgaTGACggtgaca
V$TCF11_01	953 (+)	1.000	0.880	GTCAccgtcatca
V$AP1_Q2	954 (-)	1.000	0.901	gaTGACggtga
V$AP1FJ_Q2	954 (-)	1.000	0.902	gaTGACggtga
V$CREB_02	955 (-)	1.000	0.922	ttgaTGACggtg
V$TCF11_01	959 (+)	1.000	0.986	GTCAtcaaagggg
V$AP4_Q5	967 (-)	1.000	0.860	caCAGCccct
V$DELTAEF1_01	978 (+)	1.000	0.864	gtaaACCTgaa
V$IRF1_01	986 (+)	0.765	0.863	gaaaaacCAAAcg
V$VMYB_01	992 (+)	1.000	0.899	ccaAACGgac
V$VMYB_02	992 (+)	1.000	0.932	ccaAACGga
V$DELTAEF1_01	1007 (-)	1.000	0.941	tgacACCTaca
V$AP1_Q4	1009 (-)	1.000	0.874	agTGACaccta
V$AP1FJ_Q2	1009 (-)	1.000	0.948	agTGACaccta
V$AP1_Q2	1009 (-)	1.000	0.923	agTGACaccta
V$TCF11_01	1014 (+)	1.000	0.890	GTCActtatatca
V$NKX25_01	1016 (-)	1.000	0.930	atAAGTg
V$GATA_C	1016 (-)	0.868	0.882	tGATATaagtg
V$GATA1_03	1017 (-)	1.000	0.883	cttgtGATAtaagt
V$GATA1_02	1017 (-)	1.000	0.909	cttgtGATAtaagt
V$GATA1_04	1017 (-)	1.000	0.912	ttgtGATAtaagt
V$GATA3_03	1019 (-)	0.875	0.869	tgTGATataa
V$GATA2_03	1019 (-)	1.000	0.893	tgtGATAtaa
V$LMO2COM_02	1019 (-)	1.000	0.888	gtGATAtaa

V$PADS_C	1020 (-)	0.904	0.851	tGTGATata
V$USF_Q6	1023 (+)	0.864	0.881	atCACAaggc
V$E47_02	1030 (+)	1.000	0.903	ggctaCAGGtgtcttt
V$E47_01	1031 (+)	0.833	0.907	gctACAGgtgtcttt
V$LMO2COM_01	1032 (+)	1.000	0.963	ctaCAGGtgtct
V$MYOD_01	1032 (+)	1.000	0.934	ctaCAGGtgtct
V$USF_Q6	1033 (-)	0.818	0.852	gaCACCtgta
V$MYOD_Q6	1033 (-)	1.000	0.918	gaCACCtgta
V$DELTAEF1_01	1033 (-)	1.000	0.948	agacACCTgta
V$USF_C	1034 (-)	0.856	0.922	aCACCTgt
V$HNF3B_01	1036 (+)	0.855	0.862	aggtgTCTTtatttc
V$FREAC7_01	1037 (-)	1.000	0.894	tggaaaTAAAgacacc
V$HFH3_01	1038 (+)	0.838	0.912	gtgTCTTtatttc
V$HFH8_01	1038 (+)	0.778	0.870	gtgTCTTtatttc
V$NFAT_Q6	1044 (-)	1.000	0.967	cagtgGAAAtaa
V$AHRARNT_01	1053 (-)	1.000	0.878	cagcaccagCGTGcag
V$IK2_01	1064 (+)	1.000	0.929	tgctGGGAgcgc
V$GKLF_01	1075 (-)	0.789	0.857	caagagaaggCAGG
V$GKLF_01	1079 (-)	0.937	0.864	aaggcaagagAAGG
V$NF1_Q6	1104 (-)	1.000	0.866	cggTGGCtaggtccaaag
V$AP4_Q6	1117 (-)	1.000	0.854	ggCAGCggtg
V$AP4_Q5	1117 (-)	1.000	0.916	ggCAGCggtg
V$XBP1_01	1123 (-)	1.000	0.854	gcgcttACGTgagggca
V$ARNT_01	1124 (-)	1.000	0.859	gcgcttaCGTGagggc
V$VBP_01	1127 (-)	1.000	0.923	cTTACgtgag
V$CREBP1_01	1128 (+)	1.000	0.912	tcACGTaa
V$CREBP1_01	1128 (-)	1.000	0.917	ttACGTga
V$GKLF_01	1139 (-)	0.937	0.920	aagcaaagaaAAGG
V$HNF3B_01	1139 (+)	0.855	0.861	cctttTCTTtgcttt
V$HFH3_01	1141 (+)	0.838	0.858	tttTCTTtgcttt
V$TCF11_01	1151 (-)	0.782	0.856	TTCAtccaaaaaa
V$AP1_Q2	1170 (-)	1.000	0.860	ccTGACacccc
V$AP1FJ_Q2	1170 (-)	1.000	0.906	ccTGACacccc
V$ARP1_01	1178 (-)	0.783	0.871	cgagccTCTGagccct
V$AP2_Q6	1192 (-)	0.976	0.868	ggCCCCaggccg

A total of 1223 bp was scanned.
MatInspector search was performed by Renee Chehab.

Appendix VI. List of Commonly Used Abbreviations

3′	3 prime
5′	5 prime
BCR	B Cell Receptor
bp	base pairs
cDNA	Complementary DNA
CMV	Cauliflower Mosaic Virus
°C	degrees Celsius
DNA	Deoxyribose Nucleic Acid
dH_2O	distilled water
dNTPs	deoxynucleotides
ECM	ExtraCellular Matrix
EGFP	Enhanced Green Fluorescent Protein
ELF5	E74-Like Factor 5
ESE	Epithelial Specific Ets
H_2O	water
HSA	Homo Sapiens chromosome
IFN	InterFeroN
IL	InterLeukin
kb	kilobases
LOH	Loss Of Heterozygosity
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix MetalloProteinase
mRNA	Messenger RNA

mt	mutant
PBL	Peripheral Blood Lymphocytes
PCR	Polymerase Chain Reaction
PSA	Prostate-Specific Antigen
QRT-PCR	Quantitative Reverse-Transcription PCR
RNA	Ribose Nucleic Acid
SCID	Severe Combined Immuno-Deficiency
TAD	TransActivation Domain
TCF	Ternary Complex Factor
TEB	Terminal End Bud
U	Units
UTR	UnTranslated Region
v/v	volume/volume
w/v	weight/volume
WAP	Whey Acidic Protein
wt	wildtype

Elf5 is essential for early embryogenesis and mammary gland development during pregnancy and lactation

Jiong Zhou,[1,2] Melanie A. Pritchard,[1,5] Trevor J. Wilson,[1] Erika J. Lapinskas,[1] Matthew J. Naylor,[3] Jessica Harris,[3] Sue Tsao,[1] Irene Tellis,[1] Silva Zavarsek,[1] Dakang Xu,[1] Jane Visvader,[4] Geoffrey J. Lindeman,[4] Ross Thomas,[1] Christopher J. Ormandy,[3] Paul J. Hertzog[1] and Ismail Kola[1,6]

[1]Centre for Functional Genomics and Human Disease, Monash Institute of Reproduction and Development, Monash University, Melbourne, Victoria, Australia

[3]Garvan Institute of Medical Research, Darlinghurst, NSW, Australia

[4]The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia

Running Title: *Elf5* is required for mammary and embryo development.

Present address:

[2]Prince Henry's Institute of Medical Research, Monash Medical Centre, Clayton, Australia

[6]Merck Research Laboratories, Merck &Co., Inc., Rahway, NJ, USA

[5]Corresponding author:

Centre for Functional Genomics and Human Disease, Monash Institute of Reproduction and Development, Monash University, 27-31 Wright Street, Clayton, Victoria, 3168, Australia

E-MAIL melanie.pritchard@med.monash.edu.au; FAX (613) 9594 7211

Abstract

Elf5 is an epithelial-specific ETS factor. Embryos with a null mutation in the *Elf5* gene die before embryonic day 7.5 indicating that *Elf5* is essential during mouse embryogenesis. *Elf5* is also required for the proliferation and differentiation of mouse mammary alveolar epithelial cells during pregnancy and lactation, since the loss of one functional allele leads to complete developmental arrest of the mammary gland during these stages. A quantitative mRNA expression study revealed the down-regulation of milk proteins in $Elf5^{+/-}$ pregnant and day one post partum mammary glands. A similar pregnancy-associated mammary gland developmental defect is observed in *Prolactin receptor (Prlr)* heterozygous females in which *Elf5* expression was found to be down-regulated. Mammary gland transplant experiments revealed that $Elf5^{+/-}$ mammary alveolar epithelial cells failed to develop in a wild-type mammary fat pad under the influence of pregnancy hormones, demonstrating an epithelial cell autonomous effect. Our data demonstrates that *Elf5* is a master regulator of developmental processes in the embryo and in the mammary gland during pregnancy and lactation via its action in the Prl/Prlr signalling pathway.

Key words: *ETS*/prolactin/prolactin receptor/milk protein genes

Introduction

The ETS family of transcription factors now consists of more than 50 members that share a homologous DNA binding domain. These proteins play important roles in development and disease. ETS transcription factors are involved in embryonic development, in hematopoiesis, in development of the skeletal system and in immune responses (Bassuk and Leiden, 1997; Ghysdael and Boureux, 1997; Ristevski *et al.*, 2002; Wolvetang *et al.*, 2002; Xu *et al.*, 2002). Most *ETS* genes are expressed in a wide range of cell types but recently a few that are epithelial-specific have been identified. This group includes, *ELF3* (*ESX/ESE-1/JEN/ERT*) (Andreoli *et al.*, 1997; Chang *et al.*, 1997; Choi *et al.*, 1998; Oettgen *et al.*, 1997; Tymms *et al.*, 1997), *EHF* (*ESE-3*) (Bochert *et al.*, 1998; Kas *et al.*, 2000), *PDEF* (*PSE*) (Oettgen *et al.*, 2000; Yamada *et al.*, 2000) and *ELF5* (*ESE-2*) (Zhou *et al.*, 1998; Oettgen *et al.*, 1999). ELF5, ELF3 and ESE-3 share considerable sequence similarity and are all expressed in a similar subset of epithelial tissues.

Elf3 is the most broadly expressed of the epithelial–specific *ETS* genes. It is evident in the epithelium of the gastrointestinal tract, the mammary gland, uterus and prostate, and the tongue (Andreoli *et al.*, 1997; Chang *et al.*, 1997; Oettgen *et al.*, 1997; Tymms *et al.*, 1997; unpublished data). *Elf5* and *Ese-3* have an overlapping but more restricted pattern of expression compared to *Elf3*. These genes are expressed in a subset of organs containing glandular or secretory epithelium (Oettgen *et al.*, 1999; Kas *et al.*, 2000). Although there is considerable organ overlap in the expression of *Elf5, Elf3* and *Ese-3,* several differences in their expression patterns have been observed. For instance, *Elf3* is expressed strongly in the small intestine and liver, whereas *Elf5* and *Ese-3* do not appear to be expressed in these organs at all. Both *Elf3* and *Ese-3* are expressed in the colon and pancreas, *Elf5* is not, and *Elf3* and *Elf5* are present in the stomach, whereas *Ese-3* is not.

We have previously shown that Elf5 functions as a transcription factor with similar sequence-specific DNA binding characteristics to other ETS family members (Zhou *et al.*, 1998). ELF5 (ESE-2a) is capable of transactivating a number of epithelial-specific gene promoters, including *SPRR2A, PSP* and *PSA, in vitro* (Oettgen *et al.*, 1999). In addition, recombinant ELF5 is capable of transactivating the *whey acidic protein* (*WAP*) promoter (Thomas *et al.*, 2000) in mouse mammary epithelial cells. This, together with

its expression in the mammary gland implies a function for *Elf5* in this organ. In order to establish the biological function of the *Elf5* gene in the mouse and to gain an understanding of the *ELF5* gene in human biology, we generated and characterized *Elf5* null mice.

Results

Elf5 is essential for early mouse embryogenesis

We generated a targeting construct in which part of exon 3 of the *Elf5* gene was replaced by a *NLS-LacZ-neo* cassette (Figure 1a). The targeting construct was designed to interrupt exon 3 which contains the ATG initiation codon of the Elf5 protein and produce a fusion product containing the first 28 amino acids of the Elf5 protein fused to β-galactosidase. This targeting construct was electroporated into isogenic 129SvJ J1 ES cells, and the correctly targeted *Elf5*$^{+/-}$ ES cell clones #1 and #130 were microinjected into C57Bl/6J murine blastocysts. The resulting male chimaeras were then mated with *Elf5*$^{+/+}$ C57Bl/6J female mice. Genomic Southern blot analysis and PCR screening were used to confirm the correct targeting event (Figure 1b-d). Southern analysis of tail DNA revealed a polymorphism in the wild-type *Elf5* gene locus between 129SvJ and C57Bl/6J genetic backgrounds (Figure 1c).

Heterozygotes were intercrossed to produce 513 offspring derived from the ES cell clone #130. Genotyping revealed that 34.9% were *Elf5*$^{+/+}$ and 65.1% were *Elf5*$^{+/-}$. No *Elf5*$^{-/-}$ mice were detected. Similar results were obtained for 43 adult mice derived from the ES cell clone #1 (Figure 2a). These data indicated that the *Elf5*$^{-/-}$ genotype was embryonic lethal.

Given that *Elf5*$^{-/-}$ animals were not detected among three-week-old offspring from heterozygote intercrosses, we investigated the genotypes of neonates and embryos. *Elf5*$^{-/-}$ animals were not detected among neonates nor among embryos at E13.5, E14.5 and E18.5 by Southern blot analysis. PCR analysis of postimplantation embryos dissected from the decidua at E7.5-11.5 (with the morning of vaginal plug detection corresponding to E0.5) showed that none of these embryos were *Elf5*$^{-/-}$ (Figure 2a). These results suggested that the lethality of *Elf5*$^{-/-}$ embryos was occurring before E7.5. Genomic PCR analysis of 58 blastocysts at E3.5, collected from intercrossed *Elf5*$^{+/-}$ females, detected *Elf5*$^{-/-}$ blastocysts in a ratio expected for a Mendelian distribution (Figure 2a-b). These results indicated that *Elf5*$^{-/-}$ embryos die between E3.5-7.5, during the period of implantation.

The growth of Elf5$^{-/-}$ blastocysts is defective in vitro

We investigated the growth potential of *Elf5*$^{-/-}$ blastocysts *in vitro*. Blastocysts (E3.5) from heterozygous matings were individually cultured for four days (Figure 2c). All blastocysts appeared to be morphologically normal and hatched from the zona pellucida, but unlike *Elf5*$^{+/+}$ blastocysts, *Elf5*$^{-/-}$ blastocysts failed to develop outgrowths. The *Elf5*-deficient embryos retained their blastocyst-like appearance for up to 72 hours in culture. During this time, there was a dramatic increase in size, but no obvious trophoblast giant cells formed around the *Elf5*$^{-/-}$ embryos, which was consistent with the observation that the *Elf5*$^{-/-}$ blastocysts did not attach to the culture dish. These results suggested that *Elf5*$^{-/-}$ embryos may fail to implant.

The inner cell mass (ICM) of normal blastocysts expanded rapidly in culture but in contrast the ICM of *Elf5*$^{-/-}$ blastocysts appeared not to proliferate (Figure 2c), and some of the *Elf5*$^{-/-}$ blastocysts also started to degenerate after 72 hours in culture, suggesting that *Elf5* was required for ES cell proliferation and early embryonic development.

Elf5 is expressed in the pregnant and lactating mammary gland

Earlier studies had established that the *Elf5* transcript was expressed in the mammary gland but the cell-type expression of Elf5 has never been determined. We examined the location of Elf5 protein by immunohistochemistry using an Elf5 antibody. The Elf5 protein is expressed in both ductal and alveolar epithelial cells of the day 1 post partum mammary gland in wild-type mice (Figure 3a). We also investigated the temporal expression pattern of *Elf5* mRNA in pregnant and day 1 post partum mammary glands from both *Elf5*$^{+/+}$ and *Elf5*$^{+/-}$ mice. Northern blots of poly(A)$^{+}$ mRNA were probed with the mouse *Elf5* cDNA and the two *Elf5* transcripts, *Elf5-a* (2.5 kb) and *Elf5-b* (1.5 kb) (Zhou *et al.*, 1998), were observed in both pregnant and day 1 post partum mammary glands from wild-type and heterozygous mice (Figure 3b-c). It should be noted that we did not detect any spurious *Elf5* transcripts on our Northern blots when we compared *Elf5*$^{+/+}$ and *Elf5*$^{+/-}$ mammary gland RNA, indicating that the null allele was not capable of producing alternatively spliced *Elf5* messages. Previously we showed that *Elf5* mRNA levels sharply increased between days 2 and 10 of pregnancy (Zhou *et al.*, 1998). Here we show that this surge of *Elf5* mRNA occurs after day 8 and remains at a high level throughout pregnancy (Figure 3b). The *Elf5* mRNA level in the heterozygous

day 18.5 pregnant mammary gland was reduced to ~50% of that in the corresponding *Elf5*$^{+/+}$ organs as expected, whereas the *Elf5* mRNA level in the heterozygous day 1 post partum mammary gland was reduced to ~25% of the wild-type controls (Figure 3c). This reduction in the level of *Elf5* expression resulted in a severe mammary gland developmental defect in pregnant *Elf5*$^{+/-}$ females manifest as lactational failure. The offspring from those females died as a result.

Elf5 is essential for pregnancy-associated mammary gland development

Elf5$^{+/-}$ females derived from ES cell clone #130 were fertile and gave birth to litters of morphologically normal pups whose numbers were comparable to those of *Elf5*$^{+/+}$ females. However, despite normal nursing and mothering characteristics displayed by *Elf5*$^{+/-}$ females, most of their first litter pups died within 24 hours of birth, and virtually the entire litter had perished by 48 hours. This phenomenon was never observed when *Elf5*$^{+/+}$ female littermates were mothers and was not a general occurrence among other mouse breeding stocks housed in the same animal facility. Indeed, during the backcrossing of more than 5 generations of *Elf5* heterozygous males to wild type C57Bl/6J females, we never witnessed the death of an entire litter. Occasionally the odd neonate was found dead but in our experience this is not unusual in a large animal facility. All pups were observed to attach to the nipple and suck, but newborns of *Elf5*$^{+/-}$ females died of starvation and dehydration. Lethality of the newborns was independent of their genotype (*Elf5*$^{+/+}$ or *Elf5*$^{+/-}$). Examination of one-day-old pups revealed that they lacked milk in their stomachs. These results suggested that *Elf5*$^{+/-}$ females may have a mammary gland defect.

We studied 49 *Elf5*$^{+/-}$ mothers (Figure 4). Of these, only eight supported some or all of their first litter pups. After the subsequent pregnancies of 29 of these females, loss of pups was variable and unpredictable. Four females that supported their pups after their first pregnancy, failed to support pups in subsequent pregnancies. Two *Elf5*$^{+/-}$ females derived from ES cell clone #1 were also observed for their lactating abilities. Both females failed to lactate after their first pregnancy, and pup death due to starvation and dehydration was also observed in some of their subsequent pregnancies.

Examination of whole mount mammary glands from age-matched virgin *Elf5*$^{+/+}$ and *Elf5*$^{+/-}$ females demonstrated that elongation and extension of the mammary ductal tree,

as well as ductal side branching were comparable (data not shown). Therefore, it seemed that the loss of one allele of the *Elf5* gene was affecting mammary gland development during pregnancy. In $Elf5^{+/+}$ females at 18.5 days of pregnancy, proliferation of mammary ductal epithelium and sprouting of alveolar buds was induced. Proliferation and terminal differentiation of the alveolar buds followed, resulting in fully developed lobuloalveolar structures (Figure 5a-b). In $Elf5^{+/-}$ females, increased ductal side branching and formation of the initial alveolar buds also occurred normally (Figure 5c), indicating that loss of one copy of the *Elf5* gene did not disrupt the proliferation of ductal epithelium and sprouting of alveolar buds. However, differentiation and expansion of the alveolar buds into mature lobuloalveolar mammary tissues was severely impaired in $Elf5^{+/-}$ females (Figure 5c-d). Although some proliferation and terminal differentiation of the alveolar buds in very localized regions was observed in some mammary glands in a small number of pregnant $Elf5^{+/-}$ females (data not shown), these glands were in no circumstance comparable to the fully developed wild-type glands. The mammary gland defects observed in $Elf5^{+/-}$ females remained evident after parturition, whereas full mammary growth was attained in lactating $Elf5^{+/+}$ females at one day post partum. The alveoli subunits expanded and filled with milk indicating the functional secretory state of the mammary gland (Figure 5e-f). In contrast, the alveolar buds in $Elf5^{+/-}$ mammary glands did not proliferate and differentiate, and milk was not produced in these defective glands (Figure 5g-h). Dilation of primary ducts was also impaired in some $Elf5^{+/-}$ females (data not shown). The lactational defect observed persisted in the subsequent pregnancies in $Elf5^{+/-}$ females. Whole-mount and histological examination of an 18.5 day pregnant $Elf5^{+/-}$ mammary gland during a second pregnancy revealed some alveolar development (Figure 5m-n). However, it was under developed in comparison to the wild-type glands (Figure 5i-j). The $Elf5^{+/-}$ gland at one day post partum after the second pregnancy did not undergo massive proliferation and differentiation. The alveolar structures were distended (Figure 5l) whilst the primary ducts failed to dilate (data not shown).

A study of the small number of heterozygous females that suckled pups demonstrated that the lactational performance in $Elf5^{+/-}$ females on a 129SvJ-C57Bl/6J mixed genetic background correlated with the degree of mammary gland development. This variable phenotype is probably due to a genetic modifier present in one of the genetic backgrounds. $Elf5^{+/-}$ mice were subsequently generated on a pure 129Svter genetic background, and six $Elf5^{+/-}$ females were mated with either $Elf5^{+/+}$ or $Elf5^{+/-}$ males. All

six females were incapable of lactation after multiple pregnancies (up to four pregnancies), suggesting that the mammary alveolar developmental defect was 100% penetrant in the 129Svter strain.

The Elf5$^{+/-}$ mammary defect is epithelial cell autonomous

In order to determine if the defect in ductal morphogenesis observed in *Elf5* heterozygous females was due to the lack of Elf5 in the mammary epithelium *per se* or whether the defect was secondary due to defects in other endocrine systems, we transplanted wild-type and *Elf5*$^{+/-}$ mammary epithelium into Rag1$^{-/-}$ recipient females. These females were then mated. The wild-type transplants displayed ductal branching and alveolar proliferation comparable to unmanipulated wild-type glands (Figure 6a-b). The *Elf5* heterozygous glands showed side branching but no lobuloalveolar development (Figure 6c-d), demonstrating that the *Elf5*$^{+/-}$ mammary defect was epithelial cell autonomous and not dependent on other factors.

Gene expression profiles in Elf5 and Prlr heterozygous mammary glands

The observation that a similar mammary gland developmental defect also occurs in *Prlr*$^{+/-}$ mice (Ormandy *et al.*, 1997) prompted us to examine the levels of *Elf5* in the mammary glands of *Prlr*$^{+/-}$ females. Indeed, *Elf5* was found to be down-regulated in transplanted mammary glands formed with *Prlr*$^{-/-}$ epithelium during early pregnancy (Harris *et al.* 2003). *Elf5* mRNA was dramatically reduced in the *Prlr*$^{+/-}$ day one post partum mammary gland compared to wild-type controls (Figure 7a). We also examined the expression levels of a panel of genes encoding milk proteins in the *Elf5*$^{+/-}$ glands. *WDNM1*, *WAP* and *β-casein* were all reduced (Figure 7b). This reduction was not simply due to a decreased number of epithelial cells since levels of *keratin 18*, an epithelial cell marker, remained relatively unchanged between *Elf5*$^{+/+}$ and *Elf5*$^{+/-}$ mammary glands (Figure 7b).

Discussion

Elf5 appears to be an ETS factor required for early mouse development. A number of ETS transcription factors play essential roles in mouse embryogenesis. Of the nine *ETS* family members that have been disrupted in mice, *Pu.1*$^{-/-}$, *Tel*$^{-/-}$, *Fli1*$^{-/-}$ and *Elf3*$^{-/-}$ mice were embryonic lethal around E16.5, E10.5-11.5, E11-12.5 and E11.5, respectively (Scott *et al.*, 1994; Wang *et al.*, 1997; Hart *et al.*, 2000; Spyropoulos *et al.*, 2000; Ng *et al.*, 2002). In contrast, *Ets2*$^{-/-}$ embryos died before E8.5 due to growth arrest of the ectoplacental cone and apoptosis of the embryonic ectoderm. Break down in the vascular interactions between the embryo and the maternal circulation also contributed to the *Ets2*$^{-/-}$ embryonic lethality (Yamamoto *et al.*, 1998). In this regard it is important to note that while the ETS proteins all share a similar DNA binding domain structure, have similar cognate DNA binding elements and in many cases overlapping expression, there is no functional redundancy during development.

Although the status of *Elf5* expression in the early mouse embryo and its corresponding extraembryonic tissues (before E9.5) is unknown, *Elf5* was found to be expressed in the placenta throughout E9.5-18 indicating a potential function for *Elf5* in this tissue (Zhou *et al.*, 1998). The placenta is derived from the trophoblast (Kaufman and Bard, 1999), and the trophectoderm of the blastocyst is important for the exchange of metabolites between the mother and the embryo. Therefore, the early lethality of *Elf5*$^{-/-}$ embryos may be due to the loss of *Elf5* function in the embryonic trophoblast. However, we can not rule out possible defects in the inner cell mass since the ICM of *Elf5*$^{-/-}$ blastocysts appeared to be defective in cell proliferation. Therefore, *Elf5* may be required for ES cell proliferation.

A number of epithelial-specific ETS transcription factors (ELF3, ELF5, and ESE-3) are expressed in the mammary gland, suggesting potential functions for these proteins in the mammary epithelium (Chang *et al.*, 1997; Neve *et al.*, 1998; Oettgen *et al.*, 1999; Kas *et al.*, 2000). We have shown that both ELF3 and ELF5 can transactivate a pregnancy- and lactation-associated milk protein (*WAP*) gene promoter *in vitro* (Thomas *et al.*, 2000). However, neither Elf3 nor Ese-3 is able to compensate for the lack of Elf5 in the mouse mammary gland. In this study, we have demonstrated that *Elf5* has a specific, non-redundant role in mammary gland development during pregnancy and lactation and indeed, we have demonstrated that there is a threshold level of Elf5 required since we

observed a phenotype in the heterozygous state. The study of various animal models in which the gene of interest has either been over-expressed or deleted has made it possible to assign candidate genes to one or other of the specific stages of mammary gland development; either proliferation, differentiation or involution of mammary epithelial structures. It should be noted however, that with the exceptions of *Prlr*$^{+/-}$ and *Plg*$^{+/-}$ (*plasminogen*) females (Ormandy *et al.*, 1997; Lund *et al.*, 2000), no other gene identified as being involved in mammary gland development and function, displayed a phenotype in the heterozygous state.

The mammary ductal tree can elongate and branch quickly through the entire mammary fat pad under the stimulation of ovarian hormones. Pregnancy hormones then promote alveolar proliferation, and the alveolar epithelial cells from the resulting lobuloalveolar structures eventually differentiate into secretory epithelial cells at parturition. Our results demonstrate that mammary epithelial cell proliferation and differentiation during pregnancy and the post partum period depends on a threshold level of Elf5, and that this level is unobtainable with just one functional *Elf5* allele. Death of the pups born to *Elf5*$^{+/-}$ females was due to a failure of mammary alveolar growth and the consequent failure in maternal milk production. Notably, a significant increase in *Elf5* mRNA was observed during mid-pregnancy (between days 8 and 10) when the mammary alveolar epithelial cells start to undergo a secretory differentiation process, suggesting that *Elf5* expression may trigger the onset of secretory differentiation of the mammary alveolar epithelial cells during pregnancy. Thus, an apparent correlation exists between the onset of milk protein gene-associated differentiation of the wild-type mammary alveolar epithelial cells and the expression levels of *Elf5*. Our data suggests that in the *Elf5* heterozygous mammary gland, the level of *Elf5* is insufficient to trigger the expression of genes required for subsequent differentiation and proliferation of the epithelial cells. In effect, what we observe at day 18.5 of pregnancy are cells blocked at an earlier stage of differentiation.

Although it may be argued that the phenotype observed is due to the dominant behaviour of a truncated Elf5 protein generated from the targeted allele, we believe that this is extremely unlikely. The disruption in the *Elf5* gene occurs 28 amino acids after the initiating methionine and if splicing occurred from the truncated exon 3 (skipping the *LacZ-neo* cassette) through to exons 4, 5 or 6, there would be a frameshift such that no functional domains of the Elf5 protein would be produced. Also, we do not see any

unexpected *Elf5* transcripts in Northern blots performed on our heterozygous mice. Unfortunately we were unable to extract sufficient quantities of high quality RNA from the *Elf5*$^{-/-}$ blastocysts for Northern blot analysis.

The mammary defect in *Elf5*$^{+/-}$ females closely resembled that seen in *Prlr*$^{+/-}$ females (Ormandy *et al.*, 1997). Since disruptions in either *Elf5* or *Prlr* produce a similar phenotype, it is likely that *Elf5* and *Prlr* participate in the same pathway. Indeed, ETS binding sites have been identified in the *Prlr* promoter (Hu *et al.*, 1999) so we had hypothesised that Elf5 may be the ETS factor responsible for the regulation of this gene. However, this study and the one by Harris *et al* (2003) show that *Elf5* is downstream in the prolactin signalling pathway.

Quantitative RT-PCR showed that *Elf5* expression is down-regulated in the *Prlr*$^{+/-}$ day 1 post partum mammary gland, placing Elf5 downstream in the Prl/Prlr signalling cascade. In addition, a number of other genes are either up- or down-regulated in both *Prlr*$^{+/-}$ and *Elf5*$^{+/-}$ mammary glands demonstrating that the loss of either gene affects the expression of genes in a common pathway (Harris *et al.* 2003). Significantly, the expression of three genes encoding the milk proteins, WDNM1, β-casein, and WAP, is down-regulated. The expression of *WDNM1* and *β-casein* begins around day 9 of pregnancy and that of *WAP* at day 14 (Robinson *et al.*, 1995; Kannius-Janson *et al.*, 1998), coinciding with the upsurge in *Elf5* expression. Our results suggest that Elf5 is upstream of these milk protein genes, placing it early in the transcriptional response to prolactin. The milk proteins, such as WDNM1, β-casein and WAP, are expressed at different times during pregnancy, and hence represent different mammary alveolar differentiation states. These genes are all regulated, either directly or indirectly, by Elf5, thus it is likely that the mammary gland developmental defect observed in the *Elf5*$^{+/-}$ pregnant female is due to a block in milk protein-associated alveolar differentiation in response to prolactin signalling.

Finally, our transplant studies showed that the mammary gland defect in the *Elf5*$^{+/-}$ mice is epithelial cell autonomous and not due to other influences such as systemic endocrine alterations since the *Elf5*$^{+/-}$ mammary epithelium transplanted to a wild-type mammary fat pad failed to undergo pregnancy-associated lobuloalveolar development.

Epithelial cell proliferation and differentiation is tightly controlled during mammalian development. This study has provided *in vivo* evidence of the involvement of *Elf5* in these critical developmental processes and further, defined a dual role for *Elf5*. It is initially required for early embryonic development and again later in life during development of the mammary gland in pregnancy. The role of *Elf5* in the mammary gland is extremely interesting since in the absence of one *Elf5* allele, the development of the gland during pregnancy is completely shut down.

Materials and methods

Gene Targeting

Murine *Elf5* genomic clones were isolated from a 129SvJ λFIXII genomic library (Stratagene) using a m*Elf5* cDNA probe. The targeting construct contained a 2.2 kb fragment, encoding the first 84 bp of m*Elf5* cDNA coding sequence, as the left arm and a 1.65 kb *Sal*I-*BamH*I fragment as the right arm. The homologous recombination event replaces a portion of exon 3 and intron 3 with a *NLS-LacZ-neo* cassette in which the pMC1-*neo* cassette was in a reverse orientation to that of *Elf5* transcription. A pMC1-*TK* cassette was placed 3′ of the right arm to allow for enrichment of targeted ES cells. *Hind*III digested genomic DNA was used to determine the genotype by Southern blot analysis using a 400 bp probe as indicated in Figure 1. PCR genotyping used two m*Elf5*-specific primers, P_S (5′-GCACACCCAGAATTGAAGATTCC-3′) and P_{AS1} (5′-CCTTCACTGCACGTGGACTG-3′) and one *neo*-specific primer P_{NEO} (5′-ATTCGCCAATGACAAGACGC-3′).

In vitro blastocyst outgrowth assays

Murine blastocysts were collected and cultured as described (Takai *et al.*, 2000). Blastocyst outgrowths were inspected and photographed daily. After 4 days in culture, genomic DNA was isolated and genotyped as described (Takai *et al.*, 2000).

Mammary gland whole-mounts and histology

For whole-mounts, inguinal mammary glands were fixed in Carnoy's fixative and stained in carmine alum as described (Kordon *et al.*, 1995). For histology, inguinal mammary glands were fixed in Bouin's fixative and paraffin sections (10 μm) were stained with hematoxylin and eosin

Immunohistochemistry

Immunohistochemical detection was performed using the TSA-Indirect Kit (NEN Life Science Products) according to the manufacturer's instructions. Antigen retrieval

involved treatment with 0.2% trypsin in 0.4% $CaCl_2$ for 10 min at 37°C. Endogenous peroxidases were quenched with 6% H_2O_2 in PBS for 20 min. Affinity-purified anti-Elf5 rabbit sera (Lapinskas, Palmer, Hammacher *et al.*, manuscript in preparation) was diluted 1:2500 in the blocking buffer supplied with the kit and incubated for 60 min at room temperature. Secondary antibody was a 1:200 dilution of biotinylated goat anti-rabbit IgG (DAKO) and incubation was for 40 min at room temperature. Visualization utilised the DAB substrate kit (DAKO). Sections were counterstained with Harris haematoxylin and coverslips mounted with DPX (BDH Laboratory Supplies). A second serial section on the same slide was used as a negative control, and treated with affinity-purified pre-immune sera from the same rabbit.

Mammary Epithelium Transplants

Transplants were performed as described (Brisken *et al.* 1999). Mammary gland fragments from 16-week-old *Elf5* heterozygotes and wild-type littermates were transplanted into the cleared fat pads of 3-week-old C57Bl6/Rag1$^{-/-}$ mice (Mombaerts *et al.*, 1992). The recipients were mated at 8 weeks post transplant and the transplants examined by whole-mount microscopy and histology at day 1 post partum. The whole-mounts were stained with carmine alum and the sections stained with hematoxylin and eosin.

Gene Expression Profiling

Poly(A)$^+$ mRNA was extracted from the inguinal mammary glands of *Elf5* heterozygotes and wild-type littermates at day 18.5 of pregnancy and at day 1 post partum. Two samples of each were reverse transcribed using AMV reverse transcriptase (Promega). PCR primers were designed for *Prlr*, *WDNM1*, *β-casein* and *WAP* using Macvector. To be sure the reaction was specific for cDNA and not genomic DNA the primers were designed spanning an intron. PCR primers for *Elf5* were (1) sense primer: 5′-TGGACTCCGTAACCCATAGCACCT-3′; (2) antisense primer: 5′-ATTGCTTAAGGGCTGATGGCATCG-3′. The PCR reactions were performed in a LightCycler (Roche) using 1 µl of the cDNA diluted 1:2, 5 pmol of primers and the FastStart DNA master SYBR Green I enzyme mix (Roche) in a 10 µl reaction volume. Relative quantification of the product was performed by comparing the crossing points

of different samples above background and assuming that a difference of one cycle in the linear phase of the reaction corresponds to a two fold difference in transcript levels between samples. The samples were analysed twice and the results reported as an average of both animal and analysis duplicates.

Acknowledgements

The authors would like to thank Caroline Wang and Belinda Duscio for excellent technical assistance, and Ms Jodie Palmer and Dr. Annet Hammacher for designing the peptide used to raise the ELF5 antibody.

References

Andreoli, J.M., Jang, S.I., Chung, E., Coticchia, C.M., Steinert, P.M. and Markova, N.G. (1997) The expression of a novel, epithelium-specific ets transcription factor is restricted to the most differentiated layers in the epidermis. *Nucleic Acids Res.*, **25**, 4287-4295.

Bassuk, A.G. and Leiden, J.M. (1997) The role of Ets transcription factors in the development and function of the mammalian immune system. *Adv. Immunol.*, **64**, 65-104.

Bochert, M.A., Kleinbaum, L.A., Sun, L.Y. and Burton, F.H. (1998) Molecular cloning and expression of Ehf, a new member of the ets transcription factor/oncoprotein gene family. *Biochem. Biophys. Res. Commun.*, **246**, 176-181.

Brisken, C., Kaur, S., Chavarria, T.E., Binart, N., Sutherland, R.L., Weinberg, R.A., Kelly, P.A. and Ormandy, C.J. (1999) Prolactin controls mammary gland development via direct and indirect mechanisms. *Dev. Biol.*, **210**, 96-106.

Chang, C.H., Scott, G.K., Kuo, W.L., Xiong, X., Suzdaltseva, Y., Park, J.W., Sayre, P., Erny, K., Collins, C., Gray, J.W. and Benz, C.C. (1997) ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. *Oncogene*, **14**, 1617-1622.

Choi, S.G., Yi, Y., Kim, Y.S., Kato, M., Chang, J., Chung, H.W., Hahm, K.B., Yang, H.K., Rhee, H.H., Bang, Y.J. and Kim, S.J. (1998) A novel ets-related transcription factor, ERT/ESX/ESE-1, regulates expression of the transforming growth factor-beta type II receptor. *J. Biol. Chem.*, **273**, 110-117.

Ghysdael, J. and Boureux, A. (1997) The Ets family of transcriptional regulators. In Yaniv, M. and Ghysdael, J. (ed.), *Oncogenes as Transcriptional Regulators*. Birkhauser Verlag. Basel, Switzerland, Vol. 1, pp. 22.

Harris, J., Naylor M.J., Robertson, F.G., Wittlin, S., Zhou, J., Lapinskas, E.J., Hertzog, P.J., Linderman, G.J., Visvader, J., Kelly, P., Pritchard M. and Ormandy, C.J. (2003) Transcript profiling of prolactin-dependent models of mammary development reveals the Ets transcription factor Elf-5 as a key modulator of prolactin action on the mammary epithelium (summitted).

Hart, A., Melet, F., Grossfeld, P., Chien, K., Jones, C., Tunnacliffe, A., Favier, R. and Bernstein, A. (2000) Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. *Immunity*, **13**, 167-177.

Hu, Z.Z., Zhuang, L., Meng, J., Leondires, M. and Dufau, M.L. (1999) The human prolactin receptor gene structure and alternative promoter utilization: the generic promoter hPIII and a novel human promoter hP(N). *J. Clin. Endocrinol. Metab.*, **84**, 1153-1156.

Kannius-Janson, M., Lidberg, U., Hulten, K., Gritli-Linde, A., Bjursell, G. and Nilsson, J. (1998) Studies of the regulation of the mouse carboxyl ester lipase gene in mammary gland. *Biochem. J.*, **336**, 577-585.

Kas, K., Finger, E., Grall, F., Gu, X., Akbarali, Y., Boltax, J., Weiss, A., Oettgen, P., Kapeller, R. and Libermann, T.A. (2000) ESE-3, a novel member of an epithelium-specific ets transcription factor subfamily, demonstrates different target gene specificity from ESE-1. *J. Biol. Chem.*, **275**, 2986-2998.

Kaufman, M.H. and Bard, J.B.L. (1999) *The Anatomical Basis of Mouse Development.* Academic Press. San Diego, CA, USA.

Kordon, E.C., McKnight, R.A., Jhappan, C., Hennighausen, L., Merlino, G. and Smith, G.H. (1995) Ectopic TGF beta 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. *Dev. Biol.*, **168**, 47-61.

Lund, L.R., Bjorn, S.F., Sternlicht, M.D., Nielsen, B.S., Solberg, H., Usher, P.A., Osterby, R., Christensen, I.J., Stephens, R.W., Bugge, T.H., Dano, K. and Werb, Z. (2000) Lactational competence and involution of the mouse mammary gland require plasminogen. *Development*, **127**, 4481-4492.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S. and Papaioannou, V.E. (1992) RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*, **68**, 869-877.

Neve, R., Chang, C.H., Scott, G.K., Wong, A., Friis, R.R., Hynes, N.E. and Benz, C.C. (1998) The epithelium-specific ets transcription factor ESX is associated with mammary gland development and involution. *FASEB J.*, **12**, 1541-1550.

Ng, A.Y., Waring, P., Ristevski, S., Wang, C., Wilson, T., Pritchard, M., Hertzog, P. and Kola, I. (2002) Inactivation of the transcription factor Elf3 in mice results in dysmorphogenesis and altered differentiation of intestinal epithelium. *Gastroenterology*, **122**, 1455-1466.

Oettgen, P., Alani, R.M., Barcinski, M.A., Brown, L., Akbarali, Y., Boltax, J., Kunsch, C., Munger, K. and Libermann, T.A. (1997) Isolation and characterization of a novel epithelium-specific transcription factor, ESE-1, a member of the ets family. *Mol. Cell Biol.*, **17**, 4419-4433.

Oettgen, P., Finger, E., Sun, Z., Akbarali, Y., Thamrongsak, U., Boltax, J., Grall, F., Dube, A., Weiss, A., Brown, L., Quinn, G., Kas, K., Endress, G., Kunsch, C. and Libermann, T.A. (2000) PDEF, a novel prostate epithelium-specific ets transcription factor, interacts with the androgen receptor and activates prostate-specific antigen gene expression. *J. Biol. Chem.*, **275**, 1216-1225.

Oettgen, P., Kas, K., Dube, A., Gu, X., Grall, F., Thamrongsak, U., Akbarali, Y., Finger, E., Boltax, J., Endress, G., Munger, K., Kunsch, C. and Libermann, T.A. (1999) Characterization of ESE-2, a novel ESE-1-related Ets transcription factor that is restricted to glandular epithelium and differentiated keratinocytes. *J. Biol. Chem.*, **274**, 29439-29452.

Ormandy, C.J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N. and Kelly, P.A. (1997) Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev.*, **11**, 167-178.

Ristevski, S., Tam, P.P., Hertzog, P.J. and Kola, I. (2002) Ets2 is expressed during morphogenesis of the somite and limb in the mouse embryo. *Mech. Dev.*, **116**, 165-168.

Robinson, G.W., McKnight, R.A., Smith, G.H. and Hennighausen, L. (1995) Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. *Development*, **121**, 2079-2090.

Scott, E.W., Simon, M.C., Anastasi, J. and Singh, H. (1994) Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*, **265**, 1573-1577.

Spyropoulos, D.D., Pharr, P.N., Lavenburg, K.R., Jackers, P., Papas, T.S., Ogawa, M. and Watson, D.K. (2000) Hemorrhage, impaired hematopoiesis, and lethality in mouse embryos carrying a targeted disruption of the Fli1 transcription factor. *Mol. Cell Biol.*, **20**, 5643-5652.

Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y.A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., Nakanishi, M. and Nakayama, Ki. (2000) Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. *Genes Dev.*, **14**, 1439-1447.

Thomas, R.S., Ng, A.N., Zhou, J., Tymms, M.J., Doppler, W. and Kola, I. (2000) The Elf group of Ets-related transcription factors, ELF3 and ELF5. *Adv. Exp. Med. Biol.*, **480**, 123-128.

Tymms, M.J., Ng, A.Y., Thomas, R.S., Schutte, B.C., Zhou, J., Eyre, H.J., Sutherland, G.R., Seth, A., Rosenberg, M., Papas, T., Debouck, C. and Kola, I. (1997) A novel epithelial-expressed ETS gene, ELF3: human and murine cDNA sequences, murine genomic organization, human mapping to 1q32.2 and expression in tissues and cancer. *Oncogene*, **15**, 2449-2462.

Wang, L.C., Kuo, F., Fujiwara, Y., Gilliland, D.G., Golub, T.R. and Orkin, S.H. (1997) Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. *EMBO J.*, **16**, 4374-4383.

Wolvetang, E.J., Wilson, T.J., Sanij, E., Busciglio, J., Hatzistavrou, T., Seth, A., Hertzog, P.J. and Kola, I. (2002) Ets2 overexpression in transgenic models and in down syndrome predisposes to apoptosis via the p53 pathway. *Hum. Mol. Genet.*, **12**, 247-255.

Xu, D., Wilson, T.J., Chan, D., De Luca, E., Zhou, J., Hertzog, P.J. and Kola, I. (2002) Ets1 is required for p53 transcriptional activity in UV-induced apoptosis in embryonic stem cells. *EMBO J.*, **21**, 4081-4093.

Yamada, N., Tamai, Y., Miyamoto, H. and Nozaki, M. (2000) Cloning and expression of the mouse Pse gene encoding a novel Ets family member. *Gene*, **241**, 267-274.

Yamamoto, H., Flannery, M.L., Kupriyanov, S., Pearce, J., McKercher, S.R., Henkel, G.W., Maki, R.A., Werb, Z. and Oshima, R.G. (1998) Defective trophoblast function in mice with a targeted mutation of Ets2. *Genes Dev.*, **12**, 1315-1326.

Zhou, J., NG, A.Y., Tymms, M.J., Jermiin, L.S., Seth, A.K., Thomas, R.S. and Kola, I. (1998) A novel transcription factor, ELF5, belongs to the ELF subfamily of ETS genes and maps to human chromosome 11p13-15, a region subject to LOH and rearrangement in human carcinoma cell lines. *Oncogene*, **17**, 2719-2732.

Legends to figures

Figure 1 Gene targeting of the murine *Elf5* locus. **(a)** A schematic representation of the targeting strategy. The homologous recombination event replaces a portion of exon 3 and intron 3 with a *NLS-LacZ*-neo cassette. Black boxes: Exons; exon 3 containing the ATG and exon 4 are shown, E3' represents exon 3 disrupted by insertion of the *LacZ-neo* cassette. The thin line represents plasmid sequences. H: *Hind*III; Sa: *Sal*I. **(b)** Representative Southern blot analyses of surviving ES cell clones. A 400 bp 3'-external probe recognizes a 4.6 kb wild-type and a 8.6 kb targeted *Hind*III DNA fragment, and a *neo* probe recognizes a 8.6 kb targeted *Hind*III DNA fragment. **(c)** Germ line transmission of the targeted *Elf5* allele. A 3'-external probe recognizes a 8.6 kb targeted (129SvJ), a 4.6 kb wild-type (129SvJ) and 2.0 kb wild-type (C57Bl/6J) *Hind*III bands. Left panel: Southern analysis of progeny from Chimera × C57Bl/6J; Right panel: Southern analysis of progeny from $Elf5^{+/-}$ intercrosses. **(d)** PCR analysis of progeny from $Elf5^{+/-}$ intercrosses detects a 169 bp wild-type and a 237 bp mutant band.

Figure 2 $Elf5^{-/-}$ is embryonic lethal. **(a)** Genotype distribution of $Elf5^{+/-}$ intercrosses. **(b)** Genotyping blastocysts. PCR analysis detects a 169 bp wild-type (WT) band and a 237 mutant band (KO). **(c)** Defective growth of $Elf5^{-/-}$ blastocysts *in vitro*. The length of culturing time is indicated at the top. Magnification: 200×.

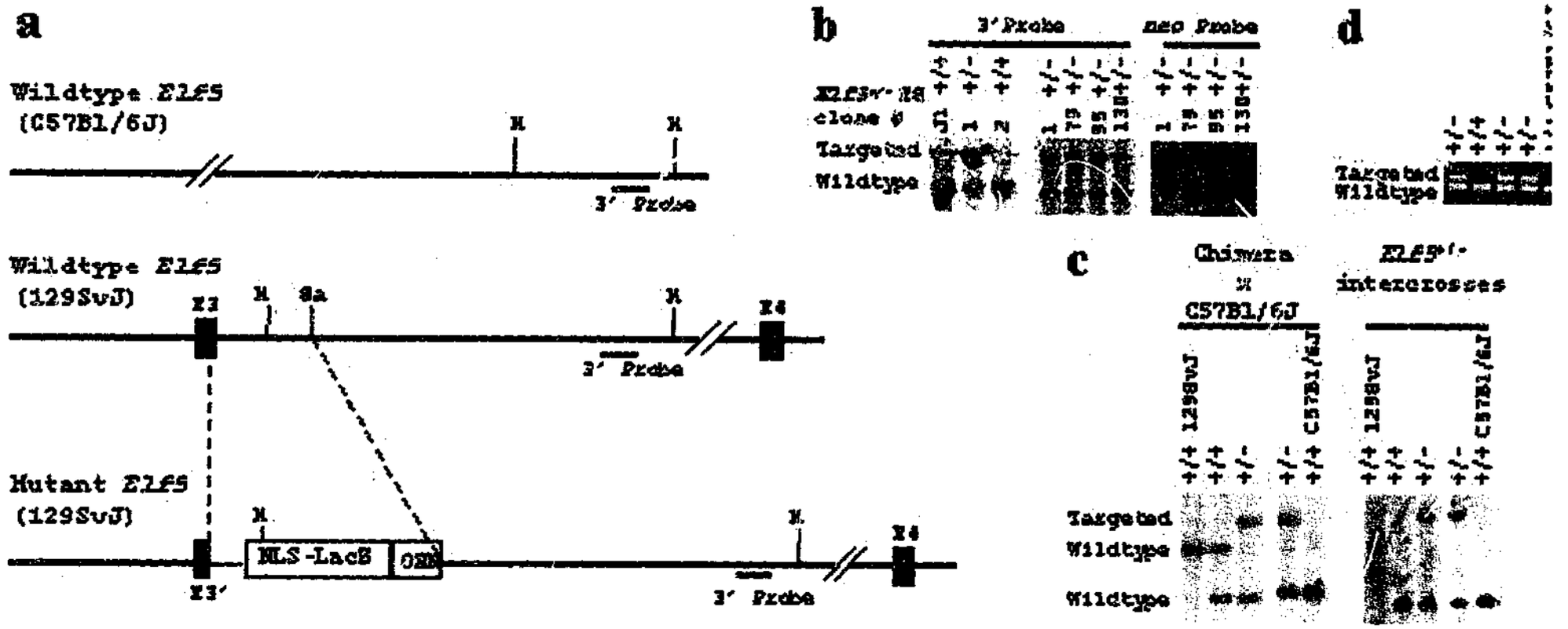
Figure 3 *Elf5* expression in $Elf5^{+/-}$ and $Elf5^{+/+}$ mammary glands. **(a)** Elf5 protein expression. Elf5 is expressed in both ductal and alveolar epithelial cells of the day 1 post partum mammary gland. Serial sections of wild-type mammary gland were treated with anti-Elf5 antibody (left panel) or with preimmune sera (right panel) and counterstained with Harris haematoxylin. **(b)** Northern blot of total RNA from wild-type mammary glands probed with a murine *Elf5* cDNA (top panel) and *18S* cDNA (lower panel). **(c)** Northern blot of Poly(A)$^+$ mRNA probed with a murine *Elf5* cDNA (top panel) and *Gapdh* cDNA (lower panel). Relative expression levels of *Elf5-b*/*Gapdh* are indicated (right panel). Lane 1: day 1 post partum $Elf5^{+/-}$ mammary gland; Lane 2: day 1 post partum $Elf5^{+/+}$ mammary gland; Lane 3: day 18.5 pregnant $Elf5^{+/-}$ mammary gland; Lane 4: day 18.5 pregnant $Elf5^{+/+}$ mammary gland.

Figure 4 $Elf5^{+/-}$ mothers, derived from $Elf5^{+/-}$ ES cell clone #130, failed to keep their pups. N/A: not applicable (these mice were culled after their first pregnancy).

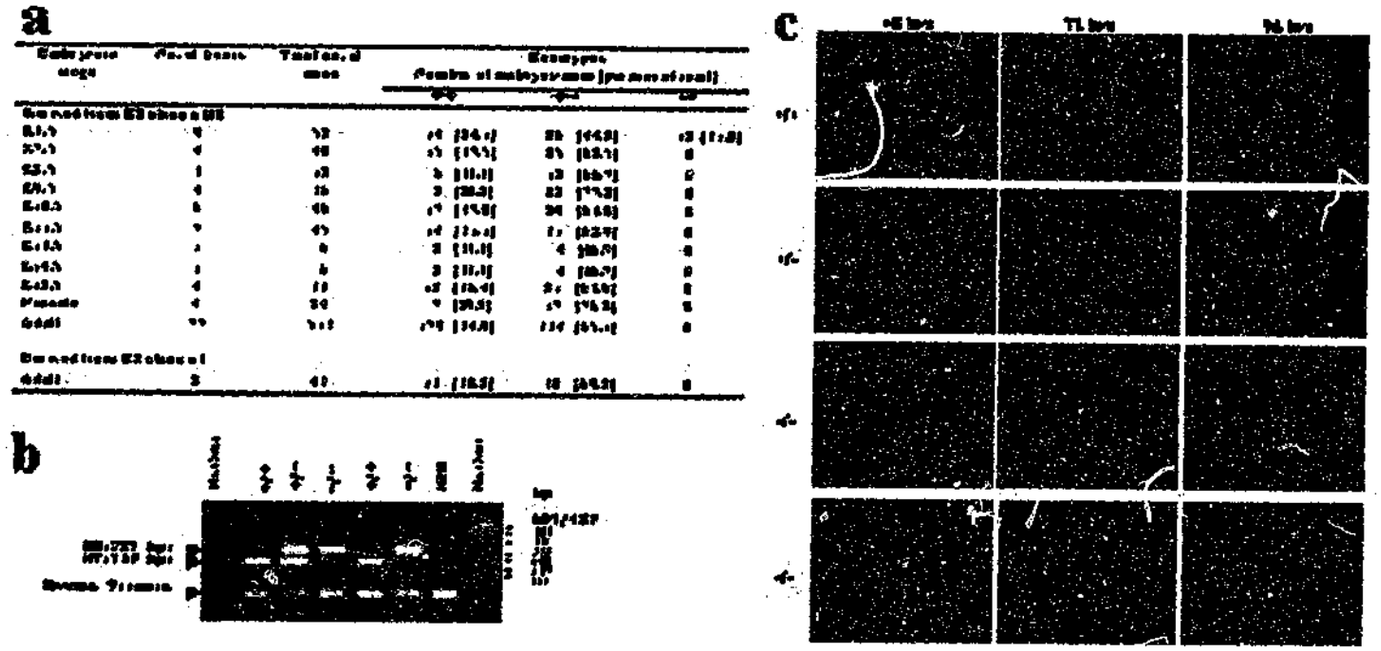
Figure 5 Mammary gland development is defective in pregnant and post partum *Elf5*$^{+/-}$ females. Whole-mount (left panels) and histological (right panels) analyses of inguinal mammary glands from a-d: 18.5 day pregnant *Elf5*$^{+/+}$ (121-day-old, a-b) and *Elf5*$^{+/-}$ (119-day-old, c-d) females during their first pregnancy; e-h: day 1 post partum *Elf5*$^{+/+}$ (127-day-old, e-f) and *Elf5*$^{+/-}$ (127-day-old, g-h) females after their first pregnancy; i-n: day 1 post partum *Elf5*$^{+/+}$ (140-day-old, i-j) and *Elf5*$^{+/-}$ (140-day-old, k-l) females, and an 18.5 day pregnant *Elf5*$^{+/-}$ (150-day-old, m-n) female after the second pregnancy. Arrows point to the alveoli. Magnification: a,c,e,g,i,k,m ×50. The bar denotes 0.05 mm (b,d,f,h,j,l,n).

Figure 6 Whole-mount analysis of wild-type and *Elf5* heterozygous mammary epithelium transplanted to a normal host mammary fat pad. Wild-type (a, b) and *Elf5*$^{+/-}$ (c, d) glands are shown. a, c whole-mount; b, d histology sections.

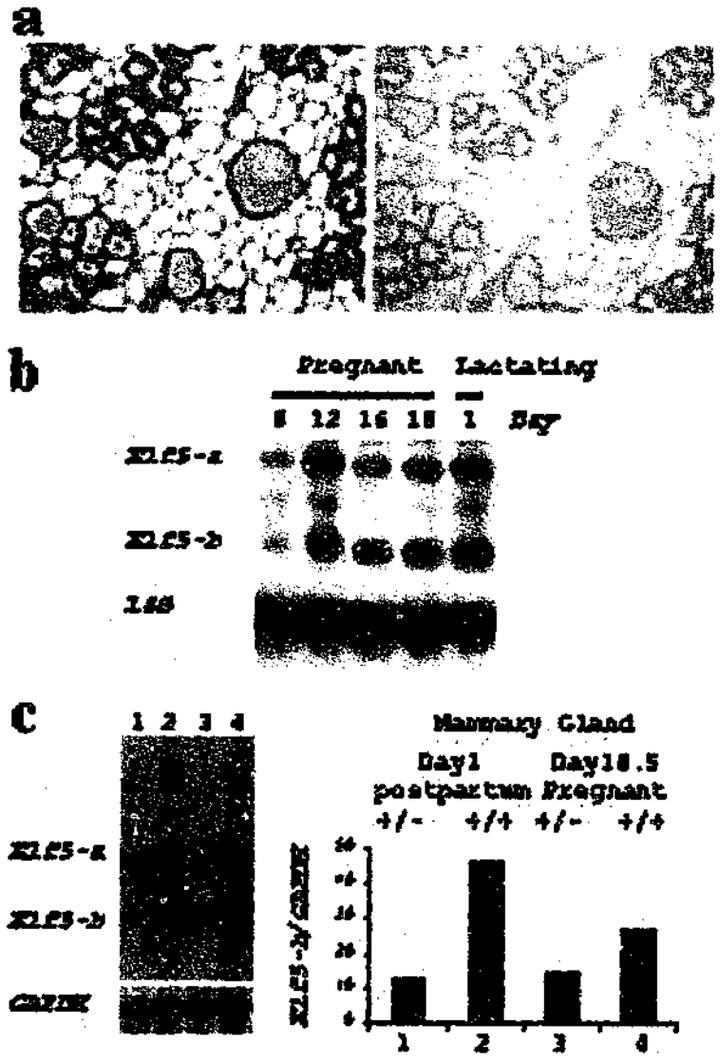
Figure 7 Gene expression profiling in *Elf5* and *Prlr* heterozygous mammary glands. **(a)** *Elf5* mRNA expression in *Prlr*$^{+/-}$ vs. *Prlr*$^{+/+}$ glands. Results are shown as numbers of transcripts for each individual mouse. **(b)** *Elf5* and milk protein gene expression in *Elf5*$^{+/-}$ vs. *Elf5*$^{+/+}$ glands. The genes tested are as indicated. The bars represent the fold increase/decrease over control wild-type glands. Lightly shaded bars represent day 18.5 of pregnancy. Darkly shaded bars represent day 1 post partum.


a
Wildtype Elf5
(C57Bl/6J)
H
H
3' Probe
Wildtype Elf5
(129SvJ)
X3
H
Sa
H
X4
3' Probe
Mutant Elf5
(129SvJ)
X3'
H
MLS-LacZ
Neo
H
X4
3' Probe
b
3' Probe
neo Probe
Elf5+/- ES clone #
Targeted
Wildtype
d
Targeted
Wildtype
c
Chimera
x
C57Bl/6J
Elf5+/-
intercrosses
129SvJ
C57Bl/6J
Targeted
Wildtype
Wildtype

Jiong Zhou



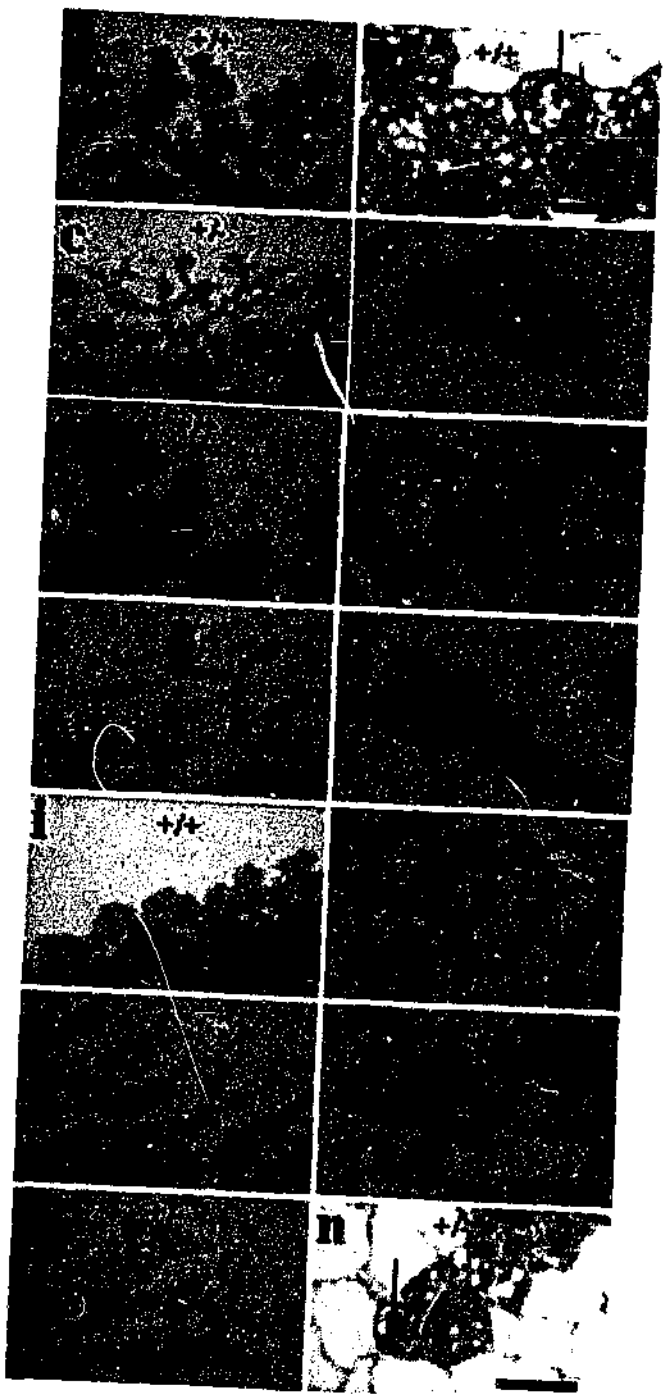
Jiong Zhou

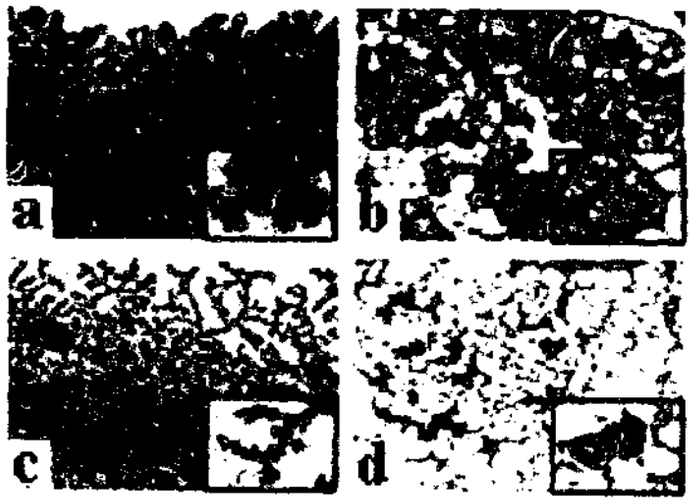

a
b
Pregnant
Lactating
8
12
16
18
1
Day
XLCS-a
XLCS-b
18S
c
1 2 3 4
Mammary Gland
Day1
postpartum
Day18.5
Pregnant
+/-
+/+
+/-
+/+
XLCS-b/GAPDH
1
2
3
4

Jiong Zhou

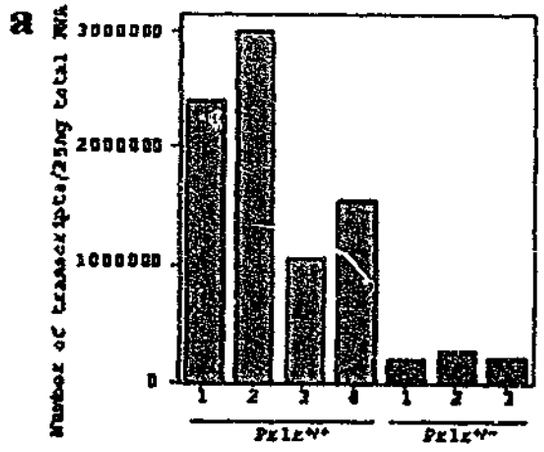
No. of Elf5+/+ mothers	First pregnancy			Subsequent pregnancies	
	All died	Some died	All survived	Death of some or entire litter	Always died
19	√			N/A	N/A
1			√	N/A	N/A
7	√				√
15	√			√	
3		√		√	
4			√	√	

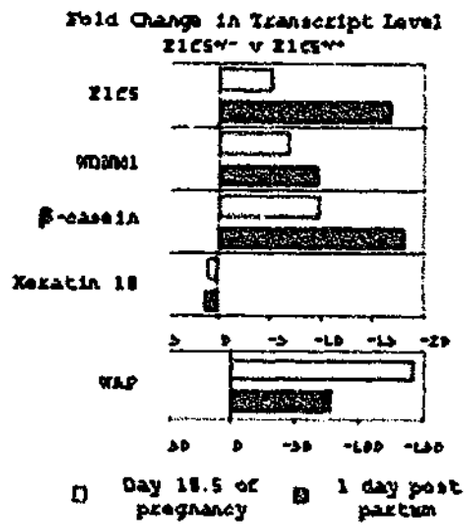
Jiong Zhou


+/+
+/+
n
+/



Jiong Zhou


a
3000000
2000000
1000000
0
1
2
3
4
1
2
3


b
Fold Change in Transcript Level
Z1C5
WDNM1
β-casein
Keratin 18
WAP
Day 18.5 of pregnancy
1 day post partum

Jiong Zhou

Reference List

Abdel-Mageed, A. B., and Agrawal, K. C. (1998). Activation of nuclear factor kappaB: potential role in metallothionein-mediated mitogenic response. Cancer Res *58*, 2335-2338.
Akira, S. (1999). Functional roles of STAT family proteins: lessons from knockout mice. Stem Cells *17*, 138-146.
Albagli, O., Klaes, A., Ferreira, E., Leprince, D., and Klambt, C. (1996). Function of ets genes is conserved between vertebrates and Drosophila. Mech Dev *59*, 29-40.
Albrecht, S., von Schweinitz, D., Waha, A., Kraus, J. A., von Deimling, A., and Pietsch, T. (1994). Loss of maternal alleles on chromosome arm 11p in hepatoblastoma. Cancer Res *54*, 5041-5044.
Alexander, C. M., Selvarajan, S., Mudgett, J., and Werb, Z. (2001). Stromelysin-1 regulates adipogenesis during mammary gland involution. J Cell Biol *152*, 693-703.
Andreoli, J. M., Jang, S. I., Chung, E., Coticchia, C. M., Steinert, P. M., and Markova, N. G. (1997). The expression of a novel, epithelium-specific ets transcription factor is restricted to the most differentiated layers in the epidermis. Nucleic Acids Res *25*, 4287-4295.
Arber, S., Ladle, D. R., Lin, J. H., Frank, E., and Jessell, T. M (2000). ETS gene Er81 controls the formation of functional connections between group 1a sensory afferents and motor neurons. Cell *101*, 485-498.
Atlas, E., Stramwasser, M., Whiskin, K., and Mueller, C. (2000). GA-binding protein alpha/beta is a critical regulator of the BRCA1 promoter. Oncogene *19*, 1933-1940.
Ayadi, A., Zheng, H., Sobieszczuk, P., Buchwalter, G., Moerman, P., Alitalo, K., and Wasylyk, B. (2001). Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1. Embo J *20*, 5139-5152.
Baert, J. L., Monte, D., Musgrove, E. A., Albagli, O., Sutherland, R. L., and de Launoit, Y. (1997). Expression of the PEA3 group of ETS-related transcription factors in human breast-cancer cells. Int J Cancer *70*, 590-597.
Baffa, R., Negrini, M., Mandes, B., Rugge, M., Ranzani, G. N., Hirohashi, S., and Croce, C. M. (1996). Loss of heterozygosity for chromosome 11 in adenocarcinoma of the stomach. Cancer Res *56*, 268-272.
Baillat, D., Begue, A., Stehelin, D., and Aumercier, M. (2002). ETS-1 transcription factor binds cooperatively to the palindromic head to head ETS-binding sites of the stromelysin-1 promoter by counteracting autoinhibition. J Biol Chem *277*, 29386-29398.
Baldassarre, G., Battista, S., Belletti, B., Thakur, S., Pentimalli, F., Trapasso, F., Fedele, M., Pierantoni, G., Croce, C. M., and Fusco, A. (2003). Negative regulation of BRCA1 gene expression by HMGA1 proteins accounts for the reduced BRCA1 protein levels in sporadic breast carcinoma. Mol Cell Biol *23*, 2225-2238.
Balmain, A. (2002). New-age tumour suppressors. Nature *417*, 235-237.
Barbeau, B., Barat, C., Bergeron, D., and Rassart, E. (1999). The GATA-1 and Spi-1 transcriptional factors bind to a GATA/EBS dual element in the Fli-1 exon 1. Oncogene *18*, 5535-5545.
Barton, K., Muthusamy, N., Fischer, C., Ting, C. N., Walunas, T. L., Lanier, L. L., and Leiden, J. M. (1998). The Ets-1 transcription factor is required for the development of natural killer cells in mice. Immunity *9*, 555-563.

Beaton, A., Broadhurst, M. K., Wilkins, R. J., and Wheeler, T. T. (2003). Suppression of beta-casein gene expression by inhibition of protein synthesis in mouse mammary epithelial cells is associated with stimulation of NF-kappaB activity and blockage of prolactin-Stat5 signaling. Cell Tissue Res *311*, 207-215.

Beitel, G. J., Tuck, S., Greenwald, I., and Horvitz, H. R. (1995). The Caenorhabditis elegans gene lin-1 encodes an ETS-domain protein and defines a branch of the vulval induction pathway. Genes Dev *9*, 3149-3162.

Bemark, M., Martensson, A., Liberg, D., and Leanderson, T. (1999). Spi-C, a novel Ets protein that is temporally regulated during B lymphocyte development. J Biol Chem *274*, 10259-10267.

Ben-David, Y., Giddens, E. B., Letwin, K., and Bernstein, A. (1991). Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. Genes Dev *5*, 908-918.

Benz, C. C., O'Hagan, R. C., Richter, B., Scott, G. K., Chang, C. H., Xiong, X., Chew, K., Ljung, B. M., Edgerton, S., Thor, A., and Hassell, J. A. (1997). HER2/Neu and the Ets transcription activator PEA3 are coordinately upregulated in human breast cancer. Oncogene *15*, 1513-1525.

Bepler, G., Fong, K. M., Johnson, B. E., O'Briant, K. C., Daly, L. A., Zimmerman, P. V., Garcia-Blanco, M. A., and Peterson, B. (1998). Association of chromosome 11 locus D11S12 with histology, stage, and metastases in lung cancer. Cancer Detect Prev *22*, 14-19.

Berclaz, G., Altermatt, H. J., Rohrbach, V., Siragusa, A., Dreher, E., and Smith, P. D. (2001). EGFR dependent expression of STAT3 (but not STAT1) in breast cancer. Int J Oncol *19*, 1155-1160.

Bertucci, F., Houlgatte, R., Granjeaud, S., Nasser, V., Loriod, B., Beaudoing, E., Hingamp, P., Jacquemier, J., Viens, P., Birnbaum, D., and Nguyen, C. (2002a). Prognosis of breast cancer and gene expression profiling using DNA arrays. Ann N Y Acad Sci *975*, 217-231.

Bertucci, F., Nasser, V., Granjeaud, S., Eisinger, F., Adelaide, J., Tagett, R., Loriod, B., Giaconia, A., Benziane, A., Devilard, E., *et al.* (2002b). Gene expression profiles of poor-prognosis primary breast cancer correlate with survival. Hum Mol Genet *11*, 863-872.

Bertucci, F., Viens, P., Hingamp, P., Nasser, V., Houlgatte, R., and Birnbaum, D. (2003). Breast cancer revisited using DNA array-based gene expression profiling. Int J Cancer *103*, 565-571.

Besnard-Guerin, C., Newsham, I., Winqvist, R., and Cavenee, W. K. (1996). A common region of loss of heterozygosity in Wilms' tumor and embryonal rhabdomyosarcoma distal to the D11S988 locus on chromosome 11p15.5. Hum Genet *97*, 163-170.

Bhat, N. K., Komschlies, K. L., Fujiwara, S., Fisher, R. J., Mathieson, B. J., Gregorio, T. A., Young, H. A., Kasik, J. W., Ozato, K., and Papas, T. S. (1989). Expression of ets genes in mouse thymocyte subsets and T cells. J Immunol *142*, 672-678.

Bidder, M., Loewy, A. P., Latifi, T., Newberry, E. P., Ferguson, G., Willis, D. M., and Towler, D. A. (2000). Ets domain transcription factor PE1 suppresses human interstitial collagenase promoter activity by antagonizing protein-DNA interactions at a critical AP1 element. Biochemistry *39*, 8917-8928.

Boccia, L. M., Lillicrap, D., Newcombe, K., and Mueller, C. R. (1996). Binding of the Ets factor GA-binding protein to an upstream site in the factor IX promoter is a critical event in transactivation. Mol Cell Biol *16*, 1929-1935.

Bochert, M. A., Kleinbaum, L. A., Sun, L. Y., and Burton, F. H. (1998). Molecular cloning and expression of Ehf, a new member of the ets transcription factor/oncoprotein gene family. Biochem Biophys Res Commun *246*, 176-181.

Bradford, A. P., Brodsky, K. S., Diamond, S. E., Kuhn, L. C., Liu, Y., and Gutierrez-Hartmann, A. (2000). The Pit-1 homeodomain and beta-domain interact with Ets-1 and modulate synergistic activation of the rat prolactin promoter. J Biol Chem *275*, 3100-3106.

Bradford, A. P., Conrad, K. E., Wasylyk, C., Wasylyk, B., and Gutierrez-Hartmann, A. (1995). Functional interaction of c-Ets-1 and GHF-1/Pit-1 mediates Ras activation of pituitary-specific gene expression: mapping of the essential c-Ets-1 domain. Mol Cell Biol *15*, 2849-2857.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248-254.

Brantley, D. M., Chen, C. L., Muraoka, R. S., Bushdid, P. B., Bradberry, J. L., Kittrell, F., Medina, D., Matrisian, L. M., Kerr, L. D., and Yull, F. E. (2001). Nuclear factor-kappaB (NF-kappaB) regulates proliferation and branching in mouse mammary epithelium. Mol Biol Cell *12*, 1445-1455.

Brar, A. K., Kessler, C. A., and Handwerger, S. (2002). An Ets motif in the proximal decidual prolactin promoter is essential for basal gene expression. J Mol Endocrinol *29*, 99-112.

Brass, A. L., Kehrli, E., Eisenbeis, C. F., Storb, U., and Singh, H. (1996). Pip, a lymphoid-restricted IRF, contains a regulatory domain that is important for autoinhibition and ternary complex formation with the Ets factor PU.1. Genes Dev *10*, 2335-2347.

Brembeck, F. H., Opitz, O. G., Libermann, T. A., and Rustgi, A. K. (2000). Dual function of the epithelial specific ets transcription factor, ELF3, in modulating differentiation. Oncogene *19*, 1941-1949.

Brisken, C., Ayyannan, A., Nguyen, C., Heineman, A., Reinhardt, F., Tan, J., Dey, S. K., Dotto, G. P., Weinberg, R. A., and Jan, T. (2002). IGF-2 is a mediator of prolactin-induced morphogenesis in the breast. Dev Cell *3*, 877-887.

Brockman, J. L., Schroeder, M. D., and Schuler, L. A. (2002). PRL activates the cyclin D1 promoter via the Jak2/Stat pathway. Mol Endocrinol *16*, 774-784.

Bromberg, J. (2000). Signal transducers and activators of transcription as regulators of growth, apoptosis and breast development. Breast Cancer Res *2*, 86-90.

Bromberg, J. F., Horvath, C. M., Besser, D., Lathem, W. W., and Darnell, J. E., Jr. (1998). Stat3 activation is required for cellular transformation by v-src. Mol Cell Biol *18*, 2553-2558.

Brown, L. A., Amores, A., Schilling, T. F., Jowett, T., Baert, J. L., de Launoit, Y., and Sharrocks, A. D. (1998). Molecular characterization of the zebrafish PEA3 ETS-domain transcription factor. Oncogene *17*, 93-104.

Brown, M. A., Nicolai, H., Howe, K., Katagiri, T., Lalani el, N., Simpson, K. J., Manning, N. W., Deans, A., Chen, P., Khanna, K. K., *et al.* (2002). Expression of a truncated Brca1 protein delays lactational mammary development in transgenic mice. Transgenic Res *11*, 467-478.

Brown, T. A., and McKnight, S. L. (1992). Specificities of protein:protein and protein:DNA interaction of GABP alpha and two newly defined ets-related proteins. Genes Dev *6*, 2502-2512.

Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H., and Klambt, C. (1994). The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. Nature *370*, 386-389.
Buijs, A., van Rompaey, L., Molijn, A. C., Davis, J. N., Vertegaal, A. C., Potter, M. D., Adams, C., van Baal, S., Zwarthoff, E. C., Roussel, M. F., and Grosveld, G. C. (2000). The MN1-TEL fusion protein, encoded by the translocation (12;22)(p13;q11) in myeloid leukemia, is a transcription factor with transforming activity. Mol Cell Biol *20*, 9281-9293.
Calo, V., Migliavacca, M., Bazan, V., Macaluso, M., Buscemi, M., Gebbia, N., and Russo, A. (2003). STAT proteins: from normal control of cellular events to tumorigenesis. J Cell Physiol *197*, 157-168.
Carlsson, R., Hjalmarsson, A., Liberg, D., Persson, C., and Leanderson, T. (2002). Genomic structure of mouse SPi-C and genomic structure and expression pattern of human SPI-C. Gene *299*, 271-278.
Carrere, S., Verger, A., Flourens, A., Stehelin, D., and Duterque-Coquillaud, M. (1998). Erg proteins, transcription factors of the Ets family, form homo, heterodimers and ternary complexes via two distinct domains. Oncogene *16*, 3261-3268.
Caubet, J. F., Gegonne, A., Stehelin, D., and Berger, R. (1986). [Abnormal localization of proto-oncogene c-ets 1 in acute leukemia with translocation t(1: 11)(q21: q23)]. C R Acad Sci III *302*, 589-591.
Chai, Y., Chipitsyna, G., Cui, J., Liao, B., Liu, S., Aysola, K., Yezdani, M., Reddy, E. S., and Rao, V. N. (2001). c-Fos oncogene regulator Elk-1 interacts with BRCA1 splice variants BRCA1a/1b and enhances BRCA1a/1b-mediated growth suppression in breast cancer cells. Oncogene *20*, 1357-1367.
Chan, D., Wilson, T. J., Xu, D., Cowdery, H. E., Sanij, E., Hertzog, P. J., and Kola, I. (2003). Transformation induced by Ewing's sarcoma associated EWS/FLI-1 is suppressed by KRAB/FLI-1. Br J Cancer *88*, 137-145.
Chang, C.-H., Scott GK, Kuo W-L, Xiong X, Suzdaltseva Y, Park JW, Sayre P, Erny K, Collins C, Gray JW and Benz CC (1997). *ESX*: a structurally unique *Ets* overexpressed early during human breast tumorigenesis. Oncogene *14*, 1617-1622.
Chang, J., LEe C, Hahm K-B, Youngsuk Y, Choi S-G and Kim S-J (2000). Over-expression of ERT (ESX/ESE-1/ELF3), an *ets*-related transcription factor, induces endogenous TGF-B type II receptor expression and restores the TGF-B signaling pathway in Hs578t human breast cancer cells. Oncogene *19*, 151-154.
Chapman, R. S., Lourenco, P. C., Tonner, E., Flint, D. J., Selbert, S., Takeda, K., Akira, S., Clarke, A. R., and Watson, C. J. (1999). Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. Genes Dev *13*, 2604-2616.
Chen, H., Nandi, A. K., Li, X., and Bieberich, C. J. (2002). NKX-3.1 interacts with prostate-derived Ets factor and regulates the activity of the PSA promoter. Cancer Res *62*, 338-340.
Chen, H., Ray-Gallet, D., Zhang, P., Hetherington, C. J., Gonzalez, D. A., Zhang, D. E., Moreau-Gachelin, F., and Tenen, D. G. (1995). PU.1 (Spi-1) autoregulates its expression in myeloid cells. Oncogene *11*, 1549-1560.
Chen, H. M., Gonzalez, D. A., Radomska, H. S., Voso, M. T., Sun, Z., Zhang, P., Zhang, D. E., and Tenen, D. G. (1998). Two promoters direct expression of the murine Spi-B gene, an Ets family transcription factor. Gene *207*, 209-218.

Chen, T., Bunting, M., Karim, F. D., and Thummel, C. S. (1992). Isolation and characterization of five Drosophila genes that encode an ets-related DNA binding domain. Dev Biol *151*, 176-191.
Chiu, J. J., Sgagias, M. K., and Cowan, K. H. (1996). interleukin 6 acts as a paracrine growth factor in human mammary carcinoma cell lines. Clin Cancer Res *2*, 215-221.
Choi, S. G., Yi, Y., Kim, Y. S., Kato, M., Chang, J., Chung, H. W., Hahm, K. B., Yang, H. K., Rhee, H. H., Bang, Y. J., and Kim, S. J. (1998). A novel ets-related transcription factor, ERT/ESX/ESE-1, regulates expression of the transforming growth factor-beta type II receptor. J Biol Chem *273*, 110-117.
Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem *162*, 156-159.
Chotteau-Lelievre, A., Desbiens, X., Pelczar, H., Defossez, P. A., and de Launoit, Y. (1997). Differential expression patterns of the PEA3 group transcription factors through murine embryonic development. Oncogene *15*, 937-952.
Chuang, C. F., and Ng, S. Y. (1994). Functional divergence of the MAP kinase pathway. ERK1 and ERK2 activate specific transcription factors. FEBS Lett *346*, 229-234.
Coffer, P., Lutticken, C., van Puijenbroek, A., Klop-de Jonge, M., Horn, F., and Kruijer, W. (1995). Transcriptional regulation of the junB promoter: analysis of STAT-mediated signal transduction. Oncogene *10*, 985-994.
Consales, C., and Arnone, M. I. (2002). Functional characterization of Ets-binding sites in the sea urchin embryo: three base pair conversions redirect expression from mesoderm to ectoderm and endoderm. Gene *287*, 75-81.
Cowley, D. O., and Graves, B. J. (2000). Phosphorylation represses Ets-1 DNA binding by reinforcing autoinhibition. Genes Dev *14*, 366-376.
Criqui-Filipe, P., Ducret, C., Maira, S. M., and Wasylyk, B. (1999). Net, a negative Ras-switchable TCF, contains a second inhibition domain, the CID, that mediates repression through interactions with CtBP and de-acetylation. Embo J *18*, 3392-3403.
Dalton, S., and Treisman, R. (1992). Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell *68*, 597-612.
Das, R., and Vonderhaar, B. K. (1996). Activation of raf-1, MEK, and MAP kinase in prolactin responsive mammary cells. Breast Cancer Res Treat *40*, 141-149.
Davidson, B., Goldberg, I., Gotlieb, W. H., Kopolovic, J., Ben-Baruch, G., and Reich, R. (2003). PEA3 Is the Second Ets Family Transcription Factor Involved in Tumor Progression in Ovarian Carcinoma. Clin Cancer Res *9*, 1412-1419.
Davis, J. N., and Roussel, M. F. (1996). Cloning and expression of the murine Elf-1 cDNA. Gene *171*, 265-269.
de Bustros, A., Nelkin, B. D., Silverman, A., Ehrlich, G., Poiesz, B., and Baylin, S. B. (1988). The short arm of chromosome 11 is a "hot spot" for hypermethylation in human neoplasia. Proc Natl Acad Sci U S A *85*, 5693-5697.
de Haro, L., and Janknecht, R. (2002). Functional analysis of the transcription factor ER71 and its activation of the matrix metalloproteinase-1 promoter. Nucleic Acids Res *30*, 2972-2979.
de la Brousse, F. C., Birkenmeier, E. H., King, D. S., Rowe, L. B., and McKnight, S. L. (1994). Molecular and genetic characterization of GABP beta. Genes Dev *8*, 1853-1865.
Delannoy-Courdent, A., Mattot, V., Fafeur, V., Fauquette, W., Pollet, I., Calmels, T., Vercamer, C., Boilly, B., Vandenbunder, B., and Desbiens, X. (1998). The expression of an

Ets1 transcription factor lacking its activation domain decreases uPA proteolytic activity and cell motility, and impairs normal tubulogenesis and cancerous scattering in mammary epithelial cells. J Cell Sci *111 (Pt 11)*, 1521-1534.
Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G., and et al. (1992). Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature *359*, 162-165.
Delgado, M. D., Gutierrez, P., Richard, C., Cuadrado, M. A., Moreau-Gachelin, F., and Leon, J. (1998). Spi-1/PU.1 proto-oncogene induces opposite effects on monocytic and erythroid differentiation of K562 cells. Biochem Biophys Res Commun *252*, 383-391.
Deneen, B., and Denny, C. T. (2001). Loss of p16 pathways stabilizes EWS/FLI1 expression and complements EWS/FLI1 mediated transformation. Oncogene *20*, 6731-6741.
Deng, C. X. (2001). Tumorigenesis as a consequence of genetic instability in Brca1 mutant mice. Mutat Res *477*, 183-189.
Denoyelle, C., Vasse, M., Korner, M., Mishal, Z., Ganne, F., Vannier, J. P., Soria, J., and Soria, C. (2001). Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits the signaling pathways involved in the invasiveness and metastatic properties of highly invasive breast cancer cell lines: an in vitro study. Carcinogenesis *22*, 1139-1148.
Deramaudt, T. B., Remy, P., and Stiegler, P. (2001). Identification of interaction partners for two closely-related members of the ETS protein family, FLI and ERG. Gene *274*, 169-177.
Diaz, M. O., Le Beau, M. M., Pitha, P., and Rowley, J. D. (1986). Interferon and c-ets-1 genes in the translocation (9;11)(p22;q23) in human acute monocytic leukemia. Science *231*, 265-267.
Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res *11*, 1475-1489.
Dillner, N. B., and Sanders, M. M. (2002). Upstream stimulatory factor (USF) is recruited into a steroid hormone-triggered regulatory circuit by the estrogen-inducible transcription factor delta EF1. J Biol Chem *277*, 33890-33894.
DiNardo, D. N., Butcher, D. T., Robinson, D. P., Archer, T. K., and Rodenhiser, D. I. (2001). Functional analysis of CpG methylation in the BRCA1 promoter region. Oncogene *20*, 5331-5340.
Dobrovic, A., and Simpfendorfer, D. (1997). Methylation of the BRCA1 gene in sporadic breast cancer. Cancer Res *57*, 3347-3350.
Domenget, C., Leprince, D., Pain, B., Peyrol, S., Li, R. P., Stehelin, D., Samarut, J., and Jurdic, P. (1992). The various domains of v-myb and v-ets oncogenes of E26 retrovirus contribute differently, but cooperatively, in transformation of hematopoietic lineages. Oncogene *7*, 2231-2241.
Donaldson, L. W., Petersen, J. M., Graves, B. J., and McIntosh, L. P. (1996). Solution structure of the ETS domain from murine Ets-1: a winged helix-turn-helix DNA binding motif. Embo J *15*, 125-134.
Dreyfus, F., Picard, F., Gisselbrecht, S., Varet, B., Tambourin, P. E., and Fichelson, S. (1989). ets-1 and ets-2 proto-oncogene expression in human leukemia cells and cell lines. Nouv Rev Fr Hematol *31*, 217-221.

Ducret, C., Maira, S. M., Dierich, A., and Wasylyk, B. (1999). The net repressor is regulated by nuclear export in response to anisomycin, UV, and heat shock. Mol Cell Biol *19*, 7076-7087.
Easton, D. F., Bishop, D. T., Ford, D., and Crockford, G. P. (1993). Genetic-linkage analysis in familial breast and ovarian-cancer - results from 214 families. The American Journal of Human Genetics *52*, 678-701.
Eckel, K. L., Tentler, J. J., Cappetta, G. J., Diamond, S. E., and Gutierrez-Hartmann, A. (2003). The epithelial-specific ETS transcription factor ESX/ESE-1/Elf-3 modulates breast cancer-associated gene expression. DNA Cell Biol *22*, 79-94.
Edelman, G. M., Meech, R., Owens, G. C., and Jones, F. S. (2000). Synthetic promoter elements obtained by nucleotide sequence variation and selection for activity. Proc Natl Acad Sci U S A *97*, 3038-3043.
Eisbacher, M., Holmes, M. L., Newton, A., Hogg, P. J., Khachigian, L. M., Crossley, M., and Chong, B. H. (2003). Protein-protein interaction between Fli-1 and GATA-1 mediates synergistic expression of megakaryocyte-specific genes through cooperative DNA binding. Mol Cell Biol *23*, 3427-3441.
Ellis, M., Davis, N., Coop, A., Liu, M., Schumaker, L., Lee, R. Y., Srikanchana, R., Russell, C. G., Singh, B., Miller, W. R., *et al.* (2002). Development and validation of a method for using breast core needle biopsies for gene expression microarray analyses. Clin Cancer Res *8*, 1155-1166.
Escalante, C., Shen, L., Escalante, M., Brass, A., Edwards, T., Singh, H., and Aggarwal, A. (2002). Crystallization and characterization of PU.1/IRF-4/DNA ternary complex. J Struct Biol *139*, 55.
Espinosa, L., Santos, S., Ingles-Esteve, J., Munoz-Canoves, P., and Bigas, A. (2002). p65-NFkappaB synergizes with Notch to activate transcription by triggering cytoplasmic translocation of the nuclear receptor corepressor N-CoR. J Cell Sci *115*, 1295-1303.
Everett, R. D., and Chambon, P. (1982). A rapid and efficient method for region- and strand-specific mutagenesis of cloned DNA. Embo J *1*, 433-437.
Fabre, A., McCann, A. H., O'Shea, D., Broderick, D., Keating, G., Tobin, B., Gorey, T., and Dervan, P. A. (1999). Loss of heterozygosity of the Wilms' tumor suppressor gene (WT1) in in situ and invasive breast carcinoma. Hum Pathol *30*, 661-665.
Feldman, R. J., Sementchenko, V. I., Gayed, M., Fraig, M. M., and Watson, D. K. (2003a). Pdef expression in human breast cancer is correlated with invasive potential and altered gene expression. Cancer Res *63*, 4626-4631.
Feldman, R. J., Sementchenko, V. I., and Watson, D. K. (2003b). The epithelial-specific Ets factors occupy a unique position in defining epithelial proliferation, differentiation and carcinogenesis. Anticancer Res *23*, 2125-2131.
Fitzsimmons, D., Lutz, R., Wheat, W., Chamberlin, H. M., and Hagman, J. (2001). Highly conserved amino acids in Pax and Ets proteins are required for DNA binding and ternary complex assembly. Nucleic Acids Res *29*, 4154-4165.
Fuh, G., and Wells, J. A. (1995). Prolactin receptor antagonists that inhibit the growth of breast cancer cell lines. J Biol Chem *270*, 13133-13137.
Fujii, Y., Shimizu, T., Kusumoto, M., Kyogoku, Y., Taniguchi, T., and Hakoshima, T. (1999). Crystal structure of an IRF-DNA complex reveals novel DNA recognition and cooperative binding to a tandem repeat of core sequences. Embo J *18*, 5028-5041.

Fujimura, Y., Yamamoto, H., Hamazato, F., and Nozaki, M. (1994). One of two Ets-binding sites in the cytokeratin EndoA enhancer is essential for enhancer activity and binds to Ets-2 related proteins. Nucleic Acids Res *22*, 613-618.
Fujiwara, S., Koizumi, S., Fisher, R. J., Bhat, N. K., and Papas, T. S. (1990). Phosphorylation of the ETS-2 protein: regulation by the T-cell antigen receptor-CD3 complex. Mol Cell Biol *10*, 1249-1253.
Futreal, P. A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L. M., Haugen-Strano, A., Swensen, J., Miki, Y., and et al. (1994). BRCA1 mutations in primary breast and ovarian carcinomas. Science *266*, 120-122.
Galetic, I., Maira, S. M., Andjelkovic, M., and Hemmings, B. A. (2003). Negative regulation of ERK and Elk by protein kinase B modulates c-Fos transcription. J Biol Chem *278*, 4416-4423.
Gao, B., Shen, X., Kunos, G., Meng, Q., Goldberg, I. D., Rosen, E. M., and Fan, S. (2001). Constitutive activation of JAK-STAT3 signaling by BRCA1 in human prostate cancer cells. FEBS Lett *488*, 179-184.
Garcia, R., Bowman, T. L., Niu, G., Yu, H., Minton, S., Muro-Cacho, C. A., Cox, C. E., Falcone, R., Fairclough, R., Parsons, S., *et al.* (2001). Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene *20*, 2499-2513.
Garret-Sinha, L. A., Dahl, T., Rao, S., Barton, K. P., and Simon, M. C. (2001). PU.1 exhibits partial functional redundancy with Spi-B, but not with Ets-1 or Elf-1. Blood *97*, 2908-2912.
Garvie, C. W., Hagman, J., and Wolberger, C. (2001). Structural studies of Ets-1/Pax5 complex formation on DNA. Mol Cell *8*, 1267-1276.
Gavrilov, D., Kenzior, O., Evans, M., Calaluce, R., and Folk, W. R. (2001). Expression of urokinase plasminogen activator and receptor in conjunction with the ets family and AP-1 complex transcription factors in high grade prostate cancers. Eur J Cancer *37*, 1033-1040.
Gegonne, A., Leprince, D., Duterque-Coquillaud, M., Vandenbunder, B., Flourens, A., Ghysdael, J., Debuire, B., and Stehelin, D. (1987). Multiple domains for the chicken cellular sequences homologous to the v-ets oncogene of the E26 retrovirus. Mol Cell Biol *7*, 806-812.
Ghadersohi, A., and Sood, A. K. (2001). Prostate epithelium-derived Ets transcription factor mRNA is overexpressed in human breast tumors and is a candidate breast tumor marker and a breast tumor antigen. Clin Cancer Res *7*, 2731-2738.
Gille, H., Sharrocks, A. D., and Shaw, P. E. (1992). Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. Nature *358*, 414-417.
Gille, H., Strahl, T., and Shaw, P. E. (1995). Activation of ternary complex factor Elk-1 by stress-activated protein kinases. Curr Biol *5*, 1191-1200.
Giovane, A., Pintzas, A., Maira, S. M., Sobieszczuk, P., and Wasylyk, B. (1994). Net, a new ets transcription factor that is activated by Ras. Genes Dev *8*, 1502-1513.
Goel, A., and Janknecht, R. (2003). Acetylation-mediated transcriptional activation of the ETS protein ER81 by p300, P/CAF, and HER2/Neu. Mol Cell Biol *23*, 6243-6254.
Goetz, T. L., Gu, T. L., Speck, N. A., and Graves, B. J. (2000). Auto-inhibition of Ets-1 is counteracted by DNA binding cooperativity with core-binding factor alpha2. Mol Cell Biol *20*, 81-90.

Goldberg, E. K., Glendening, J. M., Karanjawala, Z., Sridhar, A., Walker, G. J., Hayward, N. K., Rice, A. J., Kurera, D., Tebha, Y., and Fountain, J. W. (2000). Localization of multiple melanoma tumor-suppressor genes on chromosome 11 by use of homozygosity mapping-of-deletions analysis. Am J Hum Genet *67*, 417-431.

Golub, T. R., Barker, G. F., Bohlander, S. K., Hiebert, S. W., Ward, D. C., Brayward, R., Morgan, E., Raimondi, S. C., Rowley, J. D., and Gilliland, D. G. (1995). Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukaemia. Proc Natl Acad Sci U S A *92*, 4917-4921.

Golub, T. R., Barker, G. F., Lovett, M., and Gilliland, D. G. (1994). Fusion of PDGF receptor beta to a novel ets-like gene, tel, in chronic myelomonocytic leukaemia with t(5;12) chromosomal translocation. Cell *77*, 307-316.

Gomez, D. E., Alonso, D. F., Yoshiji, H., and Thorgeirsson, U. P. (1997). Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol *74*, 111-122.

Grall, F., Gu, X., Tan, L., Cho, J. Y., Inan, M. S., Pettit, A. R., Thamrongsak, U., Choy, B. K., Manning, C., Akbarali, Y., *et al.* (2003). Responses to the proinflammatory cytokines interleukin-1 and tumor necrosis factor alpha in cells derived from rheumatoid synovium and other joint tissues involve nuclear factor kappaB-mediated induction of the Ets transcription factor ESE-1. Arthritis Rheum *48*, 1249-1260.

Graves, B. J., and Petersen, J. M. (1998). Specificity within the ets family of transcription factors. Adv Cancer Res *75*, 1-55.

Greenall, A., Willingham, N., Cheung, E., Boam, D. S., and Sharrocks, A. D. (2001). DNA binding by the ETS-domain transcription factor PEA3 is regulated by intramolecular and intermolecular protein:protein interactions. J Biol Chem *276*, 16207-16215.

Grevin, D., Chen, J. H., Raes, M. B., Stehelin, D., Vandenbunder, B., and Desbiens, X. (1993). Involvement of the proto-oncogene c-ets 1 and the urokinase plasminogen activator during mouse implantation and placentation. Int J Dev Biol *37*, 519-529.

Grevin, D., Ung, S., Denhez, F., Dehem, M., Quatannens, B., Begue, A., Stehelin, D., and Martin, P. (1996). Structure and organization of the mouse elk1 gene. Gene *174*, 185-188.

Gri, G., Savio, D., Trinchieri, G., and Ma, X. (1998). Synergistic regulation of the human interleukin-12 p40 promoter by NFkappaB and Ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. J Biol Chem *273*, 6431-6438.

Gu, T. L., Goetz, T. L., Graves, B. J., and Speck, N. A. (2000). Auto-inhibition and partner proteins, core-binding factor beta (CBFbeta) and Ets-1, modulate DNA binding by CBFalpha2 (AML1). Mol Cell Biol *20*, 91-103.

Gu, Z., Lee, R. Y., Skaar, T. C., Bouker, K. B., Welch, J. N., Lu, J., Liu, A., Zhu, Y., Davis, N., Leonessa, F., *et al.* (2002). Association of interferon regulatory factor-1, nucleophosmin, nuclear factor-kappaB, and cyclic AMP response element binding with acquired resistance to Faslodex (ICI 182,780). Cancer Res *62*, 3428-3437.

Gudas, J. M., Nguyen, H., Li, T., and Cowan, K. H. (1995). Hormone-dependent regulation of BRCA1 in human breast cancer cells. Cancer Res *55*, 4561-4565.

Gudmundsson, J., Barkardottir, R. B., Eiriksdottir, G., Baldursson, T., Arason, A., Egilsson, V., and Ingvarsson, S. (1995). Loss of heterozygosity at chromosome 11 in breast cancer: association of prognostic factors with genetic alterations. Br J Cancer *72*, 696-701.

Guerra-Vladusic, F. K., Vladusic, E. A., Tsai, M. S., and Lupu, R. (2001). Signaling molecules implicated in heregulin induction of growth arrest and apoptosis. Oncol Rep *8*, 1203-1214.
Habelhah, H., Okada, F., Kobayashi, M., Nakai, K., Choi, S., Hamada, J., Moriuchi, T., Kaya, M., Yoshida, K., Fujinaga, K., and Hosokawa, M. (1999). Increased E1AF expression in mouse fibrosarcoma promotes metastasis through induction of MT1-MMP expression. Oncogene *18*, 1771-1776.
Haimov-Kochman, R., Lavy, Y., and Hochner-Celinkier, D. (2002). Review of risk factors for breast cancer--what's new? Harefuah *141*, 702-708, 761.
Harris, C. C., Reddel, R., Pfeifer, A., Iman, D., McMenamin, M., Trump, B. F., and Weston, A. (1991). Role of oncogenes and tumour suppressor genes in human lung carcinogenesis. IARC Sci Publ, 294-304.
Hart, A., Melet, F., Grossfeld, P., Chien, K., Jones, C., Tunnacliffe, A., Favier, R., and Bernstein, A. (2000a). Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. Immunity *13*, 167-177.
Hart, A. H., Corrick, C. M., Tymms, M. J., Hertzog, P. J., and Kola, I. (1995). Human ERG is a proto-oncogene with mitogenic and transforming activity. Oncogene *10*, 1423-1430.
Hart, A. H., Reventar, R., and Bernstein, A. (2000b). Genetic analysis of ETS genes in C. elegans. Oncogene *19*, 6400-6408.
Hashimoto, S., Nishizumi, H., Hayashi, R., Tsuboi, A., Nagawa, F., Takemori, T., and Sakano, H. (1999). Prf, a novel Ets family protein that binds to the PU.1 binding morig, is specifically expressed in restricted stages of B cell development. International Immunol *11*, 1423-1429.
Hassler, M., and Richmond, T. J. (2001). The B-box dominates SAP-1-SRF interactions in the structure of the ternary complex. Embo J *20*, 3018-3028.
Hennighausen, L., and Robinson, G. W. (2001). Signaling pathways in mammary gland development. Dev Cell *1*, 467-475.
Hewett, P. W., Nishi, K., Daft, E. L., and Murray, J. C. (2001). Selective expression of erg isoforms in human endothelial cells. Int J Biochem Cell Biol *33*, 347-355.
Higashino, F., Yoshida, K., Noumi, T., Seiki, M., and Fujinaga, K. (1995). Ets-related protein E1A-F can activate three different matrix metalloproteinase gene promoters. Oncogene *10*, 1461-1463.
Hill, C. S., Marais, R., John, S., Wynne, J., Dalton, S., and Treisman, R. (1993). Functional analysis of a growth factor-responsive transcription factor complex. Cell *73*, 395-406.
Hirschi, K. K., Xu, C. E., Tsukamoto, T., and Sager, R. (1996). Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. Cell Growth Differ *7*, 861-870.
Howe, L. R., Watanabe, O., Leonard, J., and Brown, A. M. (2003). Twist is up-regulated in response to Wnt1 and inhibits mouse mammary cell differentiation. Cancer Res *63*, 1906-1913.
Hsiao, J. R., Jin, Y. T., Tsai, S. T., Shiau, A. L., Wu, C. L., and Su, W. C. (2003). Constitutive activation of STAT3 and STAT5 is present in the majority of nasopharyngeal carcinoma and correlates with better prognosis. Br J Cancer *89*, 344-349.
Hsu, T., and Schulz, R. A. (2000). Sequence and functional properties of Ets genes in the model organism Drosophila. Oncogene *19*, 6409-6416.

Hu, C. J., Rao, S., Ramirez-Bergeron, D. L., Garret-Sinha, L. A., Gerondakis, S., Clark, M. R., and Simon, M. C. (2001). PU.1/Spi-B regulation of c-rel is essential for mature B cell survival. Immunity *15*, 545-555.
Huang, M., Page, C., Reynolds, R. K., and Lin, J. (2000). Constitutive activation of stat 3 oncogene product in human ovarian carcinoma cells. Gynecol Oncol *79*, 67-73.
Huang, X. M., Wei, S. G., and Wang, L. F. (1994). [Reversal of malignant phenotype of human hepatoma cells by antisense c-ets-2, c-myc and N-ras]. Zhonghua Zhong Liu Za Zhi *16*, 243-246.
Im, Y. H., Kim, H. T., Lee, C., Poulin, D., Welford, S., Sorensen, P. H., Denny, C. T., and Kim, S. J. (2000). EWS-FLI1, EWS-ERG, and EWS-ETV1 oncoproteins of Ewing tumor family all suppress transcription of transforming growth factor beta type II receptor gene. Cancer Res *60*, 1536-1540.
Inoue, A., Yoshida, N., Omoto, Y., Oguchi, S., Yamori, T., Kiyama. R., and Hayashi, S. (2002). Development of cDNA microarray for expression profiling of estrogen-responsive genes. J Mol Endocrinol *29*, 175-192.
Iotsova, V., Crepieux, P., Montpellier, C., Laudet, V., and Stehelin, D. (1996). TATA-less promoters of some Ets-family genes are efficiently repressed by wild-type p53. Oncogene *13*, 2331-2337.
Isola, J. J., Kallioniemi, O. P., Chu, L. W., Fuqua, S. A., Hilsenbeck, S. G., Osborne, C. K., and Waldman, F. M. (1995). Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. Am J Pathol *147*, 905-911.
Jacobs, D., Beitel, G. J., Clark, S. G., Horvitz, H. R., and Kornfeld, K. (1998). Gain-of-function mutations in the Caenorhabditis elegans lin-1 ETS gene identify a C-terminal regulatory domain phosphorylated by ERK MAP kinase. Genetics *149*, 1809-1322.
Janknecht, R. (1996). Analysis of the ERK-stimulated ETS transcription factor ER81. Mol Cell Biol *16*, 1550-1556.
Janknecht, R., Ernst, W. H., Pingoud, V., and Nordheim, A. (1993). Activation of ternary complex factor Elk-1 by MAP kinases. Embo J *12*, 5097-5104.
Janknecht, R., Monte, D., Baert, J. L., and de Launoit, Y. (1996). The ETS-related transcription factor ERM is a nuclear target of signaling cascades involving MAPK and PKA. Oncogene *13*, 1745-1754.
Janknecht, R., Taube, W., Ludecke, H. J., and Pongs, O. (1989). Characterization of a putative transcription factor gene expressed in the 20-OH-ecdysone inducible puff 74EF in Drosophila melanogaster. Nucleic Acids Res *17*, 4455-4464.
Janknecht, R., Zinck, R., Ernst, W. H., and Nordheim, A. (1994). Functional dissection of the transcription factor Elk-1. Oncogene *9*, 1273-1278.
Jeon, I. S., Davis, J. N., Braun, B. S., Sublett, J. E., Roussel, M. F., Denny, C. T., and Shapiro, D. N. (1995). A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. Oncogene *10*, 1229-1234.
Jewett, A., Wang, M. Y., Teruel, A., Poupak, Z., Bostanian, Z., and Park, N. H. (2003). Cytokine dependent inverse regulation of CD54 (ICAM1) and major histocompatibility complex class I antigens by nuclear factor kappaB in HEp2 tumor cell line: effect on the function of natural killer cells. Hum Immunol *64*, 505-520.
Jobling, A. I., Fang, Z., Koleski, D., and Tymms, M. J. (2002). Expression of the ETS transcription factor ELF3 in the retinal pigment epithelium. Invest Ophthalmol Vis Sci *43*, 3530-3537.

Jonsen, M. D., Petersen, J. M., Xu, Q. P., and Graves, B. J. (1996). Characterization of the cooperative function of inhibitory sequences in Ets-1. Mol Cell Biol *16*, 2065-2073.
Jousset, C., Carron, C., Boureux, A., Quang, C. T., Oury, C., Dusanter-Fourt, I., Charon, M., Levin, J., Bernard, O., and Ghysdael, J. (1997). A domain of TEL conserved in a subset of ETS proteins defines a specific oligomerization interface essential to the mitogenic properties of the TEL-PDGFR beta oncoprotein. Embo J *16*, 69-82.
Kanda, K., Nakayama, T., Onizuka, S., Tomioka, T., and Kanematsu, T. (2002). Expression of the Ets-1 proto-oncogene is linked to cell differentiation of human hepatocellular carcinoma. Hepatogastroenterology *49*, 747-751.
Kaneko, Y., Yoshida, K., Handa, M., Toyoda, Y., Nishihira, H., Tanaka, Y., Sasaki, Y., Ishida, S., Higashino, F., and Fujinaga, K. (1996). Fusion of an ETS-family gene, EIAF, to EWS by t(17;22)(q12;q12) chromosome translocation in an undifferentiated sarcoma of infancy. Genes Chromosomes Cancer *15*, 115-121.
Karim, F. D., Urness, L. D., Thummel, C. S., Klemsz, M. J., McKercher, S. R., Celada, A., Van Beveren, C., Maki, R. A., Gunther, C. V., Nye, J. A., and et al. (1990). The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. Genes Dev *4*, 1451-1453.
Karnik, P., Chen, P., Paris, M., Yeger, H., and Williams, B. R. (1998a). Loss of heterozygosity at chromosome 11p15 in Wilms tumours: identification of two independent regions. Oncogene *17*, 237-240.
Karnik, P., Paris, M., Williams, B. R., Casey, G., Crowe, J., and Chen, P. (1998b). Two distinct tumor suppressor loci within chromosome 11p15 implicated in breast cancer progression and metastasis. Hum Mol Genet *7*, 895-903.
Kas, K., Finger, E., Grall, F., Gu, X., Akbarali, Y., Boltax, J., Weiss, A., Oettgen, P., Kapeller, R., and Libermann, T. A. (2000). ESE-3, a novel member of an epithelium-specific ets transcription factor subfamily, demonstrates different target gene specificity from ESE-1. J Biol Chem *275*, 2986-2998.
Kaya, M., Yoshida, K., Higashino, F., Mitaka, T., Ishii, S., and Fujinaga, K. (1996). A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. Oncogene *12*, 221-227.
Kelley, K. M., Wang, H., and Ratnam, M. (2003). Dual regulation of ets-activated gene expression by SP1. Gene *307*, 87-97.
Kikuchi, E., Horiguchi, Y., Nakashima, J., Kuroda, K., Oya, M., Ohigashi, T., Takahashi, N., Shima, Y., Umezawa, K., and Murai, M. (2003). Suppression of hormone-refractory prostate cancer by a novel nuclear factor kappaB inhibitor in nude mice. Cancer Res *63*, 107-110.
Kinoshita, J., Kitamura, K., Tanaka, S., Sugimachi, K., Ishida, M., and Saeki, H. (2002). Clinical significance of PEA3 in human breast cancer. Surgery *131*, S222-225.
Kiuchi, N., Nakajima, K., Ichiba, M., Fukada, T., Narimatsu, M., Mizuno, K., Hibi, M., and Hirano, T. (1999). STAT3 is required for the gp130-mediated full activation of the c-myc gene. J Exp Med *189*, 63-73.
Klambt, C. (1993). The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. Development *117*, 163-176.
Kleinbaum, L. A., Duggan, C., Ferreira, E., Coffey, G. P., Buttice, G., and Burton, F. H. (1999). Human chromosomal localization, tissue/tumor expression, and regulatory function of the ets family gene EHF. Biochem Biophys Res Commun *264*, 119-126.

Klemsz, M., Hromas, R., Raskind, W., Bruno, E., and Hoffman, R. (1994). PE-1, a novel ETS oncogene family member, localizes to chromosome 1q21-q23. Genomics *20*, 291-294.
Klemsz, M. J., Maki, R. A., Papayannopoulou, T., Moore, J., and Hromas, R. (1993). Characterization of the ets oncogene family member, fli-1. J Biol Chem *268*, 5769-5773.
Klemsz, M. J., McKercher, S. R., Celada, A., Van Beveren, C., and Maki, R. A. (1990). The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. Cell *61*, 113-124.
Koizumi, S., Fisher, R. J., Fujiwara, S., Jorcyk, C., Bhat, N. K., Seth, A., and Papas, T. S. (1990). Isoforms of the human ets-1 protein: generation by alternative splicing and differential phosphorylation. Oncogene *5*, 675-681.
Kola, I., Brookes, S., Green, A. R., Garber, R., Tymms, M., Papas, T. S., and Seth, A. (1993). The Ets1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation. Proc Natl Acad Sci U S A *90*, 7588-7592.
Koo, S. H., Kwon, K. C., Ihm, C. H., Jeon, Y. M., Park, J. W., and Sul, C. K. (1999). Detection of genetic alterations in bladder tumors by comparative genomic hybridization and cytogenetic analysis. Cancer Genet Cytogenet *110*, 87-93.
Koshikawa, N., Giannelli, G., Cirulli, V., Miyazaki, K., and Quaranta, V. (2000). Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. J Cell Biol *148*, 615-624.
Koufos, A., Grundy, P., Morgan, K., Aleck, K. A., Hadro, T., Lampkin, B. C., Kalbakji, A., and Cavenee, W. K. (1989). Familial Wiedemann-Beckwith syndrome and a second Wilms tumor locus both map to 11p15.5. Am J Hum Genet *44*, 711-719.
Kwiatkowski, B. A., Bastian, L. S., Bauer, T. R. J., Tsai, S., Zielinska-Kwiatkowska, A. G., and Hickstein, D. D. (1998). The ets family member Tel binds to the Fli-1 oncoprotein and inhibits its transcriptional activity. J Biol Chem *273*, 17525-17530.
Kwon, Y. W., Jang, E. R., Lee, Y. M., Kim, Y. S., Kwon, K. S., Jang, H. S., Oh, C. K., and Kim, K. W. (2000). Insulin-like growth factor II induces interleukin-6 expression via NFkappaB activation in psoriasis. Biochem Biophys Res Commun *278*, 312-317.
Laemmli, U. K., Beguin, F., and Gujer-Kellenberger, G. (1970). A factor preventing the major head protein of bacteriophage T4 from random aggregation. J Mol Biol *47*, 69-85.
Laget, M. P., Defossez, P. A., Albagli, O., Baert, J. L., Dewitte, F., Stehelin, D., and de Launoit, Y. (1996). Two functionally distinct domains responsible for transactivation by the Ets family member ERM. Oncogene *12*, 1325-1336.
Lai, Z. C., and Rubin, G. M. (1992). Negative control of photoreceptor development in Drosophila by the product of the yan gene, an ETS domain protein. Cell *70*, 609-620.
Laing, M. A., Coonrod, S., Hinton, B. T., Downie, J. W., Tozer, R., Rudnicki, M. A., and Hassell, J. A. (2000). Male sexual dysfunction in mice bearing targeted mutant alleles of the PEA3 ets gene. Mol Cell Biol *20*, 9337-9345.
LaMarco, K., Thompson, C. C., Byers, B. P., Walton, E. M., and McKnight, S. L. (1991). Identification of Ets- and notch-related subunits in GA binding protein. Science *253*, 789-792.
Lamph, W. W., Wamsley, P., Sassone-Corsi, P., and Verma, I. M. (1988). Induction of proto-oncogene JUN/AP-1 by serum and TPA. Nature *334*, 629-631.

Lane, T. F., Lin, C., Brown, M. A., Solomon, E., and Leder, P. (2000). Gene replacement with the human BRCA1 locus: tissue specific expression and rescue of embryonic lethality in mice. Oncogene *19*, 4085-4090.
Le Beau, M. M., Rowley, J. D., Sacchi, N., Watson, D. K., Papas, T. S., and Diaz, M. O. (1986). Hu-ets-2 is translocated to chromosome 8 in the t(8;21) in acute myelogenous leukemia. Cancer Genet Cytogenet *23*, 269-274.
Le Gallic, L., Sgouras, D., Beal, G., Jr., and Mavrothalassitis, G. (1999). Transcriptional repressor ERF is a Ras/mitogen-activated protein kinase target that regulates cellular proliferation. Mol Cell Biol *19*, 4121-4133.
Lee, S. B., and Haber, D. A. (2001). Wilms tumor and the WT1 gene. Exp Cell Res *264*, 74-99.
Leiden, J. M., Wang, C. Y., Petryniak, B., Markovitz, D. M., Nabel, G. J., and Thompson, C. B. (1992). A novel Ets-related transcription factor, Elf-1, binds to human immunodeficiency virus type 2 regulatory elements that are required for inducible trans activation in T cells. J Virol *66*, 5890-5897.
Leprince, D., Gegonne, A., Coll, J., de Taisne, C., Schneeberger, A., Lagrou, C., and Stehelin, D. (1983). A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. Nature *306*, 395-397.
Lewin, A. R., Reid, L. E., McMahon, M., Stark, G. R., and Kerr, I. M. (1991). Molecular analysis of a human interferon-inducible gene family. Eur J Biochem *199*, 417-423.
Li, L., and Shaw, P. E. (2002). Autocrine-mediated activation of STAT3 correlates with cell proliferation in breast carcinoma lines. J Biol Chem *277*, 17397-17405.
Li, R., Pei, H., and Papas, T. (1999). The p42 variant of ETS1 protein rescues defective Fas-induced apoptosis in colon carcinoma cells. Proc Natl Acad Sci U S A *96*, 3876-3881.
Li, R., Pei, H., and Watson, D. K. (2000). Regulation of Ets function by protein - protein interactions. Oncogene *19*, 6514-6523.
Li, Z., and Li, J. J. (2001). Effector genes altered in mcf-7 human breast cancer cells after exposure to fractionated ionizing radiation. Radiat Res *155*, 543-553.
Lim, F., Kraut, N., Framptom, J., and Graf, T. (1992). DNA binding by c-Ets-1, but not v-Ets, is repressed by an intramolecular mechanism. Embo J *11*, 643-652.
Lincoln, D. W., 2nd, Phillips, P. G., and Bove, K. (2003). Estrogen-induced Ets-1 promotes capillary formation in an in vitro tumor angiogenesis model. Breast Cancer Res Treat *78*, 167-178.
Linderson, Y., Cross, D., Neurath, M. F., and Pettersson, S. (1997). NFE, a new transcriptional activator that facilitates p50 and c-Rel-dependent IgH 3' enhancer activity. Eur J Immunol *27*, 468-475.
Liu, D., Pavlopoulos, E., Modi, W., Moschonas, N., and Mavrothalassitis, G. (1997a). ERF: genomic organization, chromosomal localization and promoter analysis of the human and mouse genes. Oncogene *14*, 1445-1451.
Liu, X., Gallego, M. I., Smith, G. H., Robinson, G. W., and Hennighausen, L. (1998). Functional rescue of Stat5a-null mammary tissue through the activation of compensating signals including Stat5b. Cell Growth Differ *9*, 795-803.
Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A., and Hennighausen, L. (1997b). Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev *11*, 179-186.

Llovera, M., Pichard, C., Bernichtein, S., Jeay, S., Touraine, P., Kelly, P. A., and Goffin, V. (2000). Human prolactin (hPRL) antagonists inhibit hPRL-activated signaling pathways involved in breast cancer cell proliferation. Oncogene *19*, 4695-4705.
Lodie, T. A., Reiner, M., Coniglio, S., Viglianti, G., and Fenton, M. J. (1998). Both PU.1 and nuclear factor-kappa B mediate lipopolysaccharide- induced HIV-1 long terminal repeat transcription in macrophages. J Immunol *161*, 268-276.
Lopez, M., Oettgen, P., Akbarali, Y., Dendorfer, U., and Libermann, T. A. (1994). ERP, a new member of the ets transcription factor/oncoprotein family: cloning, characterization, and differential expression during B-lymphocyte development. Mol Cell Biol *14*, 3292-3309.
Lucas, B. K., Ormandy, C. J., Binart, N., Bridges, R. S., and Kelly, P. A. (1998). Null mutation of the prolactin receptor gene produces a defect in maternal behavior. Endocrinology *139*, 4102-4107.
Ludwig, C. U., Raefle, G., Dalquen, P., Stulz, P., Stahel, R., and Obrecht, J. P. (1991). Allelic loss on the short arm of chromosome 11 in non-small-cell lung cancer. Int J Cancer *49*, 661-665.
Ma, X. J., Salunga, R., Tuggle, J. T., Gaudet, J., Enright, E., McQuary, P., Payette, T., Pistone, M., Stecker, K., Zhang, B. M., *et al.* (2003a). Gene expression profiles of human breast cancer progression. Proc Natl Acad Sci U S A *100*, 5974-5979.
Ma, Y., Koza-Taylor, P. H., DiMattia, D. A., Hames, L., Fu, H., Dragnev, K. H., Turi, T., Beebe, J. S., Freemantle, S. J., and Dmitrovsky, E. (2003b). Microarray analysis uncovers retinoid targets in human bronchial epithelial cells. Oncogene *22*, 4924-4932.
Maida, Y., Kyo, S., Kanaya, T., Wang, Z., Yatabe, N., Tanaka, M., Nakamura, M., Ohmichi, M., Gotoh, N., Murakami, S., and Inoue, M. (2002). Direct activation of telomerase by EGF through Ets-mediated transactivation of TERT via MAP kinase signaling pathway. Oncogene *21*, 4071-4079.
Maira, S. M., Wurtz, J. M., and Wasylyk, B. (1996). Net (ERP/SAP2) one of the Ras-inducible TCFs, has a novel inhibitory domain with resemblance to the helix-loop-helix motif. Embo J *15*, 5849-5865.
Majerus, M. A., Bibollet-Ruche, F., Telliez, J. B., Wasylyk, B., and Bailleul, B. (1992). Serum, AP-1 and Ets-1 stimulate the human ets-1 promoter. Nucleic Acids Res *20*, 2699-2703.
Malewski, T., Gajewska, M., Zebrowska, T., and Zwierzchowski, L. (2002). Differential induction of transcription factors and expression of milk protein genes by prolactin and growth hormone in the mammary gland of rabbits. Growth Horm IGF Res *12*, 41-53.
Mancini, D. N., Rodenhiser, D. I., Ainsworth, P. J., O'Malley, F. P., Singh, S. M., Xing, W., and Archer, T. K. (1998). CpG methylation within the 5' regulatory region of the BRCA1 gene is tumor specific and includes a putative CREB binding site. Oncogene *16*, 1161-1169.
Maniatis, T., and Reed, R. (2002). An extensive network of coupling among gene expression machines. Nature *416*, 499-506.
Mao, S., Frank, R. C., Zhang, J., Miyazaki, Y., and Nimer, S. D. (1999). Functional and physical interactions between AML1 proteins and an ETS protein, MEF: implications for the pathogenesis of t(8;21)-positive leukaemias. Mol Cell Biol *19*, 3635-3644.

Marks, J. R., Huper, G., Vaughn, J. P., Davis, P. L., Norris, J., McDonnell, D. P., Wiseman, R. W., Futreal, P. A., and Iglehart, J. D. (1997). BRCA1 expression is not directly responsive to estrogen. Oncogene *14*, 115-121.
Marquis, S. T., Rajan, J. V., Wynshaw-Boris, A., Xu, J., Yin, G. Y., Abel, K. J., Weber, B. L., and Chodosh, L. A. (1995). The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues. Nat Genet *11*, 17-26.
Marti, A., Jehn, B., Costello, E., Keon, N., Ke, G., Martin, F., and Jaggi, R. (1994). Protein kinase A and AP-1 (c-Fos/JunD) are induced during apoptosis of mouse mammary epithelial cells. Oncogene *9*, 1213-1223.
Matrisian, L. M. (1992). The matrix-degrading metalloproteinases. Bioessays *14*, 455-463.
Matsumura, I., Kitamura, T., Wakao, H., Tanaka, H., Hashimoto, K., Albanese, C., Downward, J., Pestell, R. G., and Kanakura, Y. (1999). Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. Embo J *18*, 1367-1377.
Maus, M. V., Reilly, S. C., and Clevenger, C. V. (1999). Prolactin as a chemoattractant for human breast carcinoma. Endocrinology *140*, 5447-5450.
Mavrothalassitis, G., and Ghysdael, J. (2000). Proteins of the ETS family with transcriptional repressor activity. Oncogene *19*, 6524-6532.
McCarthy, S. A., Chen, D., Yang, B. S., Garcia Ramirez, J. J., Cherwinski, H., Chen, X. R., Klagsbrun, M., Hauser, C. A., Ostrowski, M. C., and McMahon, M. (1997). Rapid phosphorylation of Ets-2 accompanies mitogen-activated protein kinase activation and the induction of heparin-binding epidermal growth factor gene expression by oncogenic Raf-1. Mol Cell Biol *17*, 2401-2412.
McKercher, S. R., Torbett, B. E., Anderson, K. L., Henkel, G. W., Vestal, D. J., Baribault, H., Klemsz, M., Feeney, A. J., Wu, G. E., Paige, C. J., and Maki, R. A. (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. Embo J *15*, 5647-5658.
Mitas, M., Mikhitarian, K., Hoover, L., Lockett, M. A., Kelley, L., Hill, A., Gillanders, W. E., and Cole, D. J. (2002). Prostate-Specific Ets (PSE) factor: a novel marker for detection of metastatic breast cancer in axillary lymph nodes. Br J Cancer *86*, 899-904.
Miyazaki, Y., Sun, X., Uchida, H., Zhang, J., and Nimer, S. (1996). MEF, a novel transcription factor with an Elf-1 like DNA binding domain but distinct transcriptional activating properties. Oncogene *13*, 1721-1729.
Mizushima, S., and Nagata, S. (1990). pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res *18*, 5322.
Mo, Y., Ho, W., Johnston, K., and Marmorstein, R. (2001). Crystal structure of a ternary SAP-1/SRF/c-fos SRE DNA complex. J Mol Biol *314*, 495-506.
Monte, D., Baert, J. L., Defossez, P. A., de Launoit, Y., and Stehelin, D. (1994). Molecular cloning and characterization of human ERM, a new member of the Ets family closely related to mouse PEA3 and ER81 transcription factors. Oncogene *9*, 1397-1406.
Monte, D., Coutte, L., Baert, J. L., Angeli, I., Stehelin, D., and de Launoit, Y. (1995). Molecular characterization of the ets-related human transcription factor ER81. Oncogene *11*, 771-779.
Moreau-Gachelin, F., Ray, D., Mattei, M. G., Tambourin, P., and Tavitian, A. (1989). The putative oncogene Spi-1: murine chromosomal localization and transcriptional activation in murine acute erythroleukemias. Oncogene *4*, 1449-1456.

Moreau-Gachelin, F., Tavitian, A., and Tambourin, P. (1988). Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. Nature *331*, 277-280.
Morishima, A., Ohkubo, N., Maeda, N., Miki, T., and Mitsuda, N. (2003). NFkappa B regulates plasma apolipoprotein A-I and HDL-cholesterol through inhibition of PPARalpha. J Biol Chem.
Nakadate, H., Yokomori, K., Watanabe, N., Tsuchiya, T., Namiki, T., Kobayshi, H., Suita, S., Tsunematsu, Y., Horikoshi, Y., Hatae, Y., *et al.* (2001). Mutations/deletions of the WT1 gene, loss of heterozygosity on chromosome arms 11p and 11q, chromosome ploidy and histology in Wilms' tumors in Japan. Int J Cancer *94*, 396-400.
Nakayama, T., Ito, M., Ohtsuru, A., Naito, S., Nakashima, M., Fagin, J. A., Yamashita, S., and Sekine, I. (1996). Expression of the Ets-1 proto-oncogene in human gastric carcinoma: correlation with tumor invasion. Am J Pathol *149*, 1931-1939.
Neumark, E., Sagi-Assif, O., Shalmon, B., Ben-Baruch, A., and Witz, I. P. (2003). Progression of mouse mammary tumors: MCP-1-TNFalpha cross-regulatory pathway and clonal expression of promalignancy and antimalignancy factors. Int J Cancer *106*, 879-886.
Neve, R., Chang, C. H., Scott, G. K., Wong, A., Friis, R. R., Hynes, N. E., and Benz, C. C. (1998). The epithelium-specific ets transcription factor ESX is associated with mammary gland development and involution. Faseb J *12*, 1541-1550.
Neve, R. M., Ylstra, B., Chang, C. H., Albertson, D. G., and Benz, C. C. (2002). ErbB2 activation of ESX gene expression. Oncogene *21*, 3934-3938.
Ng, A. Y., Waring, P., Ristevski, S., Wang, C., Wilson, T., Pritchard, M., Hertzog, P., and Kola, I. (2002). Inactivation of the transcription factor Elf3 in mice results in dysmorphogenesis and altered differentiation of intestinal epithelium. Gastroenterology *122*, 1455-1466.
Nguyen, V. T., and Benveniste, E. N. (2000). Involvement of STAT-1 and ets family members in interferon-gamma induction of CD40 transcription in microglia/macrophages. J Biol Chem *275*, 23674-23684.
Nozaki, M., Onishi, Y., Kanno, N., Ono, Y., and Fujimura, Y. (1996). Molecular cloning of Elk-3, a new member of the Ets family expressed during mouse embryogenesis and analysis of its transcriptional repression activity. DNA Cell Biol *15*, 855-862.
Nozawa, M., Yomogida, K., Kanno, N., Nonomura, N., Miki, T., Okuyama, A., Nishimune, Y., and Nozaki, M. (2000). Prostate-specific transcription factor hPSE is translated only in normal prostate epithelial cells. Cancer Res *60*, 1348-1352.
Nunn, M., Weiher, H., Bullock, P., and Duesberg, P. (1984). Avian erythroblastosis virus E26: nucleotide sequence of the tripartite onc gene and of the LTR, and analysis of the cellular prototype of the viral ets sequence. Virology *139*, 330-339.
Nunn, M. F., Seeburg, P. H., Moscovici, C., and Duesberg, P. H. (1983). Tripartite structure of the avian erythroblastosis virus E26 transforming gene. Nature *306*, 391-395.
Nye, J. A., Petersen, J. M., Gunther, C. V., Jonsen, M. D., and Graves, B. J. (1992). Interaction of murine ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. Genes Dev *6*, 975-990.
Oettgen, P., Akbarali, Y., Boltax, J., Best, J., Kunsch, C., and Libermann, T. A. (1996). Characterization of NERF, a novel transcription factor related to the Ets factor ELF-1. Mol Cell Biol *16*, 5091-5106.
Oettgen, P., Alani, R. M., Barcinski, M. A., Brown, L., Akbarali, Y., Boltax, J., Kunsch, C., Munger, K., and Libermann, T. A. (1997a). Isolation and characterization of a novel

epithelium-specific transcription factor, ESE-1, a member of the ets family. Mol Cell Biol *17*, 4419-4433.
Oettgen, P., Barcinski, M., Boltax, J., Stolt, P., Akbarali, Y., and Libermann, T. A. (1999a). Genomic organization of the human ELF3 (ESE-1/ESX) gene, a member of the Ets transcription factor family, and identification of a functional promoter. Genomics *55*, 358-362.
Oettgen, P., Carter, K. C., Augustus, M., Barcinski, M., Boltax, J., Kunsch, C., and Libermann, T. A. (1997b). The Novel Epithelial-Specific Ets Transcription Factor Gene ESX Maps to Human Chromosome 1q32.1. Genomics *445*, 456-457.
Oettgen, P., Finger, E., Sun, Z., Akbarali, Y., Thamrongsak, U., Boltax, J., Grall, F., Dube, A., Weiss, A., Brown, L., *et al.* (2000). PDEF, a novel prostate epithelium-specific ets transcription factor, interacts with the androgen receptor and activates prostate-specific antigen gene expression. J Biol Chem *275*, 1216-1225.
Oettgen, P., Kas, K., Dube, A., Gu, X., Grall, F., Thamrongsak, U., Akbarali, Y., Finger, E., Boltax, J., Endress, G., *et al.* (1999b). Characterization of ESE-2, a novel ESE-1-related Ets transcription factor that is restricted to glandular epithelium and differentiated keratinocytes. J Biol Chem *274*, 29439-29452.
Oka, T., Rairkar, A., and Chen, J. H. (1991). Structural and functional analysis of the regulatory sequences of the ets-1 gene. Oncogene *6*, 2077-2083.
Ouyang, L., Jacob, K. K., and Stanley, F. M. (1996). GABP mediates insulin-increased prolactin gene transcription. J Biol Chem *271*, 10425-10428.
Papas, T. S., Watson, D. K., Sacchi, N., O'Brien, S. J., and Ascione, R. (1986). Molecular evolution of ets genes from avians to mammals and their cytogenetic localization to regions involved in leukemia. Gene Amplif Anal *4*, 207-238.
Paratore, C., Brugnoli, G., Lee, H., Suter, U., and Sommer, L. (2002). The role of the ets domain transcription factor erm in modulating differentiation of neural crest stem cells. Dev Biol *250*, 168.
Park, S. H., Kim, Y. S., Park, B. K., Hougaard, S., and Kim, S. J. (2001). Sequence-specific enhancer binding protein is responsible for the differential expression of ERT/ESX/ELF-3/ESE-1/jen gene in human gastric cancer cell lines: Implication for the loss of TGF-beta type II receptor expression. Oncogene *20*, 1235-1245.
Paumelle, R., Tulasne, D., Kherrouche, Z., Plaza, S., Leroy, C., Reveneau, S., Vandenbunder, B., Fafeur, V., and Tulashe, D. (2002). Hepatocyte growth factor/scatter factor activates the ETS1 transcription factor by a RAS-RAF-MEK-ERK signaling pathway. Oncogene *21*, 2309-2319.
Peng, B., Sutherland, K. D., Sum, E. Y., Olayioye, M., Wittlin, S., Tang, T. K., Lindeman, G. J., and Visvader, J. E. (2002). CPAP is a novel stat5-interacting cofactor that augments stat5-mediated transcriptional activity. Mol Endocrinol *16*, 2019-2033.
Pereira, R., Quang, C. T., Lesault, I., Dolznig, H., Beug, H., and Ghysdael, J. (1999). FLI-1 inhibits differentiation and induces proliferation of primary erythroblasts. Oncogene *18*, 1597-1608.
Perou, C. M., Jeffrey, S. S., van de Rijn, M., Rees, C. A., Eisen, M. B., Ross, D. T., Pergamenschikov, A., Williams, C. F., Zhu, S. X., Lee, J. C., *et al.* (1999). Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc Natl Acad Sci U S A *96*, 9212-9217.

Pestell, R. G., Albanese, C., Watanabe, G., Lee, R. J., Lastowiecki, P., Zon, L., Ostrowski, M., and Jameson, J. L. (1996). Stimulation of the P-450 side chain cleavage enzyme (CYP11A1) promoter through ras- and Ets-2-signaling pathways. Mol Endocrinol *10*, 1084-1094.

Peter, M., Couturier, J., Pacquement, H., Michon, J., Thomas, G., Magdelenat, H., and Delattre, O. (1997). A new member of the ETS family fused to EWS in Ewing tumors. Oncogene *14*, 1159-1164.

Petersen, J. M., Skalicky, J. J., Donaldson, L. W., McIntosh, L. P., Alber, T., and Graves, B. J. (1995). Modulation of transcription factor Ets-1 DNA binding: DNA-induced unfolding of an alpha helix. Science *269*, 1866-1869.

Pognonec, P., Boulukos, K. E., Bosselut, R., Boyer, C., Schmitt-Verhulst, A. M., and Ghysdael, J. (1990). Identification of a Ets1 variant protein unaffected in its chromatin and in vitro DNA binding capacities by T cell antigen receptor triggering and intracellular calcium rises. Oncogene *5*, 603-610.

Poirel, H., Oury, C., Carron, C., Duprez, E., Laabi, Y., Tsapis, A., Romana, S. P., Mauchauffe, M., Le Coniat, M., Berger, R., *et al.* (1997). The TEL gene products: nuclear phosphoproteins with DNA binding properties. Oncogene *14*, 349-357.

Potter, M. D., Buijs, A., Kreider, B., van Rompaey, L., and Grosveld, G. C. (2000). Identification and characterization of a new human ETS-family transcription factor, TEL2, that is expressed in hematopoietic tissues and can associate with TEL1/ETV6. Blood *95*, 3341-3348.

Pribyl, L. J., Watson, D. K., Schulz, R. A., and Papas, T. S. (1991). D-elg, a member of the Drosophila ets gene family: sequence, expression and evolutionary comparison. Oncogene *6*, 1175-1183.

Queva, C., Leprince, D., Stehelin, D., and Vandenbunder, B. (1993). p54c-ets-1 and p68c-ets-1, the two transcription factors encoded by the c-ets-1 locus, are differentially expressed during the development of the chick embryo. Oncogene *8*, 2511-2520.

Ramana, C. V., Gil, M. P., Schreiber, R. D., and Stark, G. R. (2002). Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. Trends Immunol *23*, 96-101.

Rameil, P., Lecine, P., Ghysdael, J., Gouilleux, F., Kahn-Perles, B., and Imbert, J. (2000). IL-2 and long-term T cell activation induce physical and functional interaction between STAT5 and ETS transcription factors in human T cells. Oncogene *19*, 2086-2097.

Ramirez-Solis, R., Liu, P., and Bradley, A. (1995). Chromosome engineering in mice. Nature *378*, 720-724.

Rao, M. K., Maiti, S., Ananthaswamy, H. N., and Wilkinson, M. F. (2002). A highly active homeobox gene promoter regulated by Ets and Sp1 family members in normal granulosa cells and diverse tumor cell types. J Biol Chem *277*, 26036-26045.

Rao, V. N., Huebner, K., Isobe, M., ar-Rushdi, A., Croce, C. M., and Reddy, E. S. (1989). elk, tissue-specific ets-related genes on chromosomes X and 14 near translocation breakpoints. Science *244*, 66-70.

Ray, D., Bosselut, R., Ghysdael, J., Mattei, M. G., Tavitian, A., and Moreau-Gachelin, F. (1992). Characterization of Spi-B, a transcription factor related to the putative oncoprotein Spi-1/PU.1. Mol Cell Biol *12*, 4297-4304.

Rayet, B., and Gelinas, C. (1999). Aberrant rel/nfkb genes and activity in human cancer. Oncogene *18*, 6938-6947.

Reddy, E. S., Rao, V. N., and Papas, T. S. (1987). The erg gene: a human gene related to the ets oncogene. Proc Natl Acad Sci U S A *84*, 6131-6135.
Reddy, S. P., Vuong, H., and Adiseshaiah, P. (2003). Interplay between Proximal and Distal Promoter Elements Is Required for Squamous Differentiation Marker Induction in the Bronchial Epithelium: ROLE FOR ESE-1, Sp1, AND AP-1 PROTEINS. J Biol Chem *278*, 21378-21387.
Reeve, A. E., Sih, S. A., Raizis, A. M., and Feinberg, A. P. (1989). Loss of allelic heterozygosity at a second locus on chromosome 11 in sporadic Wilms' tumor cells. Mol Cell Biol *9*, 1799-1803.
Rice, J. C., and Futscher, B. W. (2000). Transcriptional repression of BRCA1 by aberrant cytosine methylation, histone hypoacetylation and chromatin condensation of the BRCA1 promoter. Nucleic Acids Res *28*, 3233-3239.
Rice, J. C., Massey-Brown, K. S., and Futscher, B. W. (1998). Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. Oncogene *17*, 1807-1812.
Rice, J. C., Ozcelik, H., Maxeiner, P., Andrulis, I., and Futscher, B. W. (2000). Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. Carcinogenesis *21*, 1761-1765.
Ritchie, K. A., Aprikyan, A. A., Bowen-Pope, D. F., Norby-Slycord, C. J., Conyers, S., Bartelmez, S., Sitnicka, E. H., and Hickstein, D. D. (1999). The Tel-PDGFRbeta fusion gene produces a chronic myeloproliferative syndrome in transgenic mice. Leukemia *13*, 1790-1803.
Rivera, R. R., Stuiver, M. H., Steenbergen, R., and Murre, C. (1993). Ets proteins: new factors that regulate immunoglobulin heavy-chain gene expression. Mol Cell Biol *13*, 7163-7169.
Rosen, G. D., Barks, J. L., Iademarco, M. F., Fisher, R. J., and Dean, D. C. (1994). An intricate arrangement of binding sites for the Ets family of transcription factors regulates activity of the alpha 4 integrin gene promoter. J Biol Chem *269*, 15652-15660.
Ruan, X. Z., Varghese, Z., Powis, S. H., and Moorhead, J. F. (1999). Human mesangial cells express inducible macrophage scavenger receptor. Kidney Int *56*, 440-451.
Russell, P. A., Pharoah, P. D., De Foy, K., Ramus, S. J., Symmonds, I., Wilson, A., Scott, I., Ponder, B. A., and Gayther, S. A. (2000). Frequent loss of BRCA1 mRNA and protein expression in sporadic ovarian cancers. Int J Cancer *87*, 317-321.
Sacchi, N., Watson, D. K., Guerts van Kessel, A. H., Hagemeijer, A., Kersey, J., Drabkin, H. D., Patterson, D., and Papas, T. S. (1986). Hu-ets-1 and Hu-ets-2 genes are transposed in acute leukemias with (4;11) and (8;21) translocations. Science *231*, 379-382.
Saeki, H., Oda, S., Kawaguchi, H., Ohno, S., Kuwano, H., Maehara, Y., and Sugimachi, K. (2002). Concurrent overexpression of Ets-1 and c-Met correlates with a phenotype of high cellular motility in human esophageal cancer. Int J Cancer *98*, 8-13.
Sanij, E., Hatzistavrou, T., Hertzog, P., Kola, I., and Wolvetang, E. J. (2001). Ets-2 is induced by oxidative stress and sensitizes cells to H(2)O(2)-induced apoptosis: implications for Down's syndrome. Biochem Biophys Res Commun *287*, 1003-1008.
Schaber, J. D., Fang, H., Xu, J., Grimley, P. M., and Rui, H. (1998). Prolactin activates Stat1 but does not antagonize Stat1 activation and growth inhibition by type I interferons in human breast cancer cells. Cancer Res *58*, 1914-1919.

Schlehofer, B., Pommer, W., Mellemgaard, A., Stewart, J. H., McCredie, M., Niwa, S., Lindblad, P., Mandel, J. S., McLaughlin, J. K., and Wahrendorf, J. (1996). International renal-cell-cancer study. VI. the role of medical and family history. Int J Cancer *66*, 723-726.

Schuur, E. R., and Baluda, M. A. (1991). Proto-oncogene expression in avian hematopoietic tissues. Oncogene *6*, 1409-1415.

Schweppe, R. E., and Gutierrez-Hartmann, A. (2001). Pituitary Ets-1 and GABP bind to the growth factor regulatory sites of the rat prolactin promoter. Nucleic Acids Res *29*, 1251-1260.

Scott, E. W., Simon, M. C., Anastasi, J., and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science *265*, 1573-1577.

Seidel, J. J., and Graves, B. J. (2002). An ERK2 docking site in the Pointed domain distinguishes a subset of ETS transcription factors. Genes Dev *16*, 127-137.

Seth, A., and Papas, T. S. (1990). The c-ets-1 proto-oncogene has oncogenic activity and is positively autoregulated. Oncogene *5*, 1761-1767.

Sgouras, D. N., Athanasiou, M. A., Beal, G. J., Jr., Fisher, R. J., Blair, D. G., and Mavrothalassitis, G. J. (1995). ERF: an ETS domain protein with strong transcriptional repressor activity, can suppress ets-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. Embo J *14*, 4781-4793.

Shaw, M. E., and Knowles, M. A. (1995). Deletion mapping of chromosome 11 in carcinoma of the bladder. Genes Chromosomes Cancer *13*, 1-8.

Shaw-Bruha, C. M., Pirrucello, S. J., and Shull, J. D. (1997). Expression of the prolactin gene in normal and neoplastic human breast tissues and human mammary cell lines: promoter usage and alternative mRNA splicing. Breast Cancer Res Treat *44*, 243-253.

Sheng, S., Carey, J., Seftor, E. A., Dias, L., Hendrix, M. J., and Sager, R. (1996). Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. Proc Natl Acad Sci U S A *93*, 11669-11674.

Shepherd, T. G., Kockeritz, L., Szrajber, M. R., Muller, W. J., and Hassell, J. A. (2001). The pea3 subfamily ets genes are required for HER2/Neu-mediated mammary oncogenesis. Curr Biol *11*, 1739-1748.

Shipman, R., Schraml, P., Colombi, M., and Ludwig, C. U. (1998). Allelic deletion at chromosome 11p13 defines a tumour suppressor region between the catalase gene and D11S935 in human non-small cell lung carcinoma. Int J Oncol *12*, 107-111.

Shipman, R., Schraml, P., Colombi, M., Raefle, G., and Ludwig, C. U. (1993). Loss of heterozygosity on chromosome 11p13 in primary bladder carcinoma. Hum Genet *91*, 455-458.

Simpson, S., Woodworth, C. D., and DiPaolo, J. A. (1997). Altered expression of Erg and Ets-2 transcription factors is associated with genetic changes at 21q22.2-22.3 in immortal and cervical carcinoma cell lines. Oncogene *14*, 2149-2157.

Skalicky, J. J., Donaldson, L. W., Petersen, J. M., Graves, B. J., and McIntosh, L. P. (1996). Structural coupling of the inhibitory regions flanking the ETS domain of murine Ets-1. Protein Sci *5*, 296-309.

Slupsky, C. M., Gentile, L. N., Donaldson, L. W., Mackereth, C. D., Seidel, J. J., Graves, B. J., and McIntosh, L. P. (1998). Structure of the Ets-1 pointed domain and mitogen-activated protein kinase phosphorylation site. Proc Natl Acad Sci U S A *95*, 12129-12134.

Smith, J. L., Schaffner, A. E., Hofmeister, J. K., Hartman, M., Wei, G., Forsthoefel, D., Hume, D. A., and Ostrowski, M. C. (2000). ets-2 is a target for an akt (Protein kinase B)/jun N-terminal kinase signaling pathway in macrophages of motheaten-viable mutant mice. Mol Cell Biol *20*, 8026-8034.
Socolovsky, M., Fallon, A. E., Wang, S., Brugnara, C., and Lodish, H. F. (1999). Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell *98*, 181-191.
Sorensen, P. H., Lessnick, S. L., Lopez-Terrada, D., Liu, X. F., Triche, T. J., and Denny, C. T. (1994). A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. Nat Genet *6*, 146-151.
Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., *et al.* (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A *100*, 8418-8423.
Spillman, M. A., and Bowcock, A. M. (1996). BRCA1 and BRCA2 mRNA levels are coordinately elevated in human breast cancer cells in response to estrogen. Oncogene *13*, 1639-1645.
Stapleton, D., Balan, I., Pawson, T., and Sicheri, F. (1999). The crystal structure of an Eph receptor SAM domain reveals a mechanism for modular dimerization. Nat Struct Biol *6*, 44-49.
Sternlicht, M. D., Lochter, A., Sympson, C. J., Huey, B., Rougier, J. P., Gray, J. W., Pinkel, D., Bissell, M. J., and Werb, Z. (1999). The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell *98*, 137-146.
Stratton, M. R., and Wooster, R. (1996). Hereditary predisposition to breast cancer. Curr Opin Genet Dev *6*, 93-97.
Su, G. H., Chen, H. M., Muthusamy, N., Garrett-Sinha, L. A., Baunoch, D., Tenen, D. G., and Simon, M. C. (1997). Defective B cell receptor-mediated responses in mice lacking the Ets protein, Spi-B. Embo J *16*, 7118-7129.
Su, G. H., Ip, H. S., Cobb, B. S., Lu, M. M., Chen, H. M., and Simon, M. C. (1996). The Ets protein Spi-B is expressed exclusively in B cells and T cells during development. J Exp Med *184*, 203-214.
Sumarsono, S. H., Wilson, T. J., Tymms, M. J., Venter, D. J., Corrick, C. M., Kola, R., Lahoud, M. J., Papas, T. S., Seth, A., and Kola, I. (1996). Down's syndrome-like skeletal abnormalities in Ets2 transgenic mice. Nature *379*, 534-537.
Swiatkowski, S., Seifert, H. H., Steinhoff, C., Prior, A., Thievessen, I., Schliess, F., and Schulz, W. A. (2003). Activities of MAP-kinase pathways in normal uroepithelial cells and urothelial carcinoma cell lines. Exp Cell Res *282*, 48-57.
Takai, N., Miyazaki, T., Nishida, M., Nasu, K., and Miyakawa, I. (2002). c-Ets1 is a promising marker in epithelial ovarian cancer. Int J Mol Med *9*, 287-292.
Teglund, S., McKay, C., Schuetz, E., van Deursen, J. M., Stravopodis, D., Wang, D., Brown, M., Bodner, S., Grosveld, G., and Ihle, J. N. (1998). Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell *93*, 841-850.
Thomas, R. S., Ng, A. N., Zhou, J., Tymms, M. J., Doppler, W., and Kola, I. (2000). The Elf group of Ets-related transcription factors. ELF3 and ELF5. Adv Exp Med Biol *480*, 123-128.

Thomas, R. S., Tymms, M. J., McKinlay, L. H., Shannon, M. F., Seth, A., and Kola, I. (1997). ETS1, NFkappaB and AP1 synergistically transactivate the human GM-CSF promoter. Oncogene *14*, 2845-2855.
Thompson, C. B., Wang, C. Y., Ho, I. C., Bohjanen, P. R., Petryniak, B., June, C. H., Miesfeldt, S., Zhang, L., Nabel, G. J., Karpinski, B., and et al. (1992). cis-acting sequences required for inducible interleukin-2 enhancer function bind a novel Ets-related protein, Elf-1. Mol Cell Biol *12*, 1043-1053.
Thompson, M. E., Jensen, R. A., Obermiller, P. S., Page, D. L., and Holt, J. T. (1995). Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet *9*, 444-450.
Tran, Y. K., and Newsham, I. F. (1996). High-density marker analysis of 11p15.5 in non-small cell lung carcinomas reveals allelic deletion of one shared and one distinct region when compared to breast carcinomas. Cancer Res *56*, 2916-2921.
Treisman, R., Marais, R., and Wynne, J. (1992). Spatial flexibility in ternary complexes between SRF and its accessory proteins. Embo J *11*, 4631-4640.
Trimble, M. S., Xin, J. H., Guy, C. T., Muller, W. J., and Hassell, J. A. (1993). PEA3 is overexpressed in mouse metastatic mammary adenocarcinomas. Oncogene *8*, 3037-3042.
Tsutsumi, S., Kuwano, H., Asao, T., Nagashima, K., Shimura, T., and Mochiki, E. (2000). Expression of Ets-1 angiogenesis-related protein in gastric cancer. Cancer Lett *160*, 45-50.
Tugores, A., Le, J., Sorokina, I., Snijders, A. J., Duyao, M., Reddy, P. S., Carlee, L., Ronshaugen, M., Mushegian, A., Watanaskul, T., *et al.* (2001). The epithelium-specific ETS protein EHF/ESE-3 is a context-dependent transcriptional repressor downstream of MAPK signaling cascades. J Biol Chem *276*, 20397-20406.
Tymms, M. J., Ng, A. Y., Thomas, R. S., Schutte, B. C., Zhou, J., Eyre, H. J., Sutherland, G. R., Seth, A., Rosenberg, M., Papas, T., *et al.* (1997). A novel epithelial-expressed ETS gene, ELF3: human and murine cDNA sequences, murine genomic organization, human mapping to 1q32.2 and expression in tissues and cancer. Oncogene *15*, 2449-2462.
Udy, G. B., Towers, R. P., Snell, R. G., Wilkins, R. J., Park, S. H., Ram, P. A., Waxman, D. J., and Davey, H. W. (1997). Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc Natl Acad Sci U S A *94*, 7239-7244.
Urano, F., Umezawa, A., Hong, W., Kikuchi, H., and Hata, J. (1996). A novel chimera gene between EWS and E1A-F, encoding the adenovirus E1A enhancer-binding protein, in extraosseous Ewing's sarcoma. Biochem Biophys Res Commun *219*, 608-612.
Vandamme, B., Lissens, W., Amfo, K., De Sutter, P., Bourgain, C., Vamos, E., and De Greve, J. (1992). Deletion of chromosome 11p13-11p15.5 sequences in invasive human ovarian cancer is a subclonal progression factor. Cancer Res *52*, 6646-6652.
Varela, L. M., Stangle-Castor, N. C., Shoemaker, S. F., Shea-Eaton, W. K., and Ip, M. M. (2001). TNFalpha induces NFkappaB/p50 in association with the growth and morphogenesis of normal and transformed rat mammary epithelial cells. J Cell Physiol *188*, 120-131.
Velculescu, V. E., and El-Deiry, W. S. (1996). Biological and clinical importance of the p53 tumor suppressor gene. Clin Chem *42*, 858-868.
Verger, A., Buisine, E., Carrere, S., Wintjens, R., Flourens, A., Coll, J., Stehelin, D., and Duterque-Coquillaud, M. (2001). Identification of amino acid residues in the ETS transcription factor Erg that mediate Erg-Jun/Fos-DNA ternary complex formation. J Biol Chem *276*, 17181-17189.

Vidarsson, H., Mikaelsdottir, E. K., Rafnar, T., Bertwistle, D., Ashworth, A., Eyfjord, J. E., and Valgeirsdottir, S. (2002). BRCA1 and BRCA2 bind Stat5a and suppress its transcriptional activity. FEBS Lett *532*, 247-252.
Viel, A., Giannini, F., Tumiotto, L., Sopracordevole, F., Visentin, M. C., and Boiocchi, M. (1992). Chromosomal localisation of two putative 11p oncosuppressor genes involved in human ovarian tumours. Br J Cancer *66*, 1030-1036.
Virmani, A. K., Fong, K. M., Kodagoda, D., McIntire, D., Hung, J., Tonk, V., Minna, J. D., and Gazdar, A. F. (1998). Allelotyping demonstrates common and distinct patterns of chromosomal loss in human lung cancer types. Genes Chromosomes Cancer *21*, 308-319.
Visser, M., Sijmons, C., Bras, J., Arceci, R. J., Godfried, M., Valentijn, L. J., Voute, P. A., and Baas, F. (1997). Allelotype of pediatric rhabdomyosarcoma. Oncogene *15*, 1309-1314.
Walunas, T. L., Wang, B., Wang, C. R., and Leiden, J. M. (2000). Cutting edge: the Ets1 transcription factor is required for the development of NK T cells in mice. J Immunol *164*, 2857-2860.
Wang, C. Y., Petryniak, B., Ho, I. C., Thompson, C. B., and Leiden, J. M. (1992). Evolutionarily conserved Ets family members display distinct DNA binding specificities. J Exp Med *175*, 1391-1399.
Wang, L., Walia, B., Evans, J., Gewirtz, A. T., Merlin, D., and Sitaraman, S. V. (2003). IL-6 induces NF-kappaB activation in the intestinal epithelia. J Immunol *171*, 3194-3201.
Wang, L. C., Kuo, F., Fujiwara, Y., Gilliland, D. G., Golub, T. R., and Orkin, S. H. (1997). Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. Embo J *16*, 4374-4383.
Wang, L. C., Swat, W., Fujiwara, Y., Davidson, L., Visvader, J., Kuo, F., Alt, F. W., Gilliland, D. G., Golub, T. R., and Orkin, S. H. (1998). The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow. Genes Dev *12*, 2392-2402.
Wang, Y., Lu, J., Lee, R., Gu, Z., and Clarke, R. (2002). Iterative normalization of cDNA microarray data. IEEE Trans Inf Technol Biomed *6*, 29-37.
Warnecke, P. M., Stirzaker, C., Song, J., Grunau, C., Melki, J. R., and Clark, S. J. (2002). Identification and resolution of artifacts in bisulfite sequencing. Methods *27*, 101-107.
Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998). Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem Sci *23*, 213-216.
Wasylyk, B., Hahn, S. L., and Giovane, A. (1993). The Ets family of transcription factors. Eur J Biochem *211*, 7-18.
Wasylyk, C., Gutman, A., Nicholson, R., and Wasylyk, B. (1991). The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. Embo J *10*, 1127-1134.
Wasylyk, C., Kerckaert, J. P., and Wasylyk, B. (1992). A novel modulator domain of Ets transcription factors. Genes Dev *6*, 965-974.
Watanabe, G., Nishimori, H., Irifune, H., Sasaki, Y., Ishida, S., Zembutsu, H., Tanaka, T., Kawaguchi, S., Wada, T., Hata, J., *et al.* (2003). Induction of tenascin-C by tumor-specific EWS-ETS fusion genes. Genes Chromosomes Cancer *36*, 224-232.
Watanabe, H., Sawada, J., Yano, K., Yamaguchi, K., Goto, M., and Handa, H. (1993). cDNA cloning of transcription factor E4TF1 subunits with Ets and notch motifs. Mol Cell Biol *13*, 1385-1391.

Watson, C. J. (2001). Stat transcription factors in mammary gland development and tumorigenesis. J Mammary Gland Biol Neoplasia *6*, 115-127.
Watson, C. J., and Burdon, T. G. (1996). Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway. Rev Reprod *1*, 1-5.
Watson, D. K., McWilliams, M. J., Lapis, P., Lautenberger, J. A., Schweinfest, C. W., and Papas, T. S. (1988). Mammalian ets-1 and ets-2 genes encode highly conserved proteins. Proc Natl Acad Sci U S A *85*, 7862-7866.
Watson, D. K., McWilliams-Smith, M. J., Kozak, C., Reeves, R., Gearhart, J., Nunn, M. F., Nash, W., Fowle, J. R., 3rd, Duesberg, P., Papas, T. S., and et al. (1986a). Conserved chromosomal positions of dual domains of the ets protooncogene in cats, mice, and humans. Proc Natl Acad Sci U S A *83*, 1792-1796.
Watson, D. K., McWilliams-Smith, M. J., Nunn, M. F., Duesberg, P. H., O'Brien, S. J., and Papas, T. S. (1985). The ets sequence from the transforming gene of avian erythroblastosis virus, E26, has unique domains on human chromosomes 11 and 21: both loci are transcriptionally active. Proc Natl Acad Sci U S A *82*, 7294-7298.
Watson, D. K., Sacchi, N., McWilliams-Smith, M. J., O'Brien, S. J., and Papas, T. S. (1986b). The avian and mammalian ets genes: molecular characterization, chromosome mapping, and implication in human leukemia. Anticancer Res *6*, 631-636.
Watson, D. K., Smyth, F. E., Thompson, D. M., Cheng, J. Q., Testa, J. R., Papas, T. S., and Seth, A. (1992). The ERGB/Fli-1 gene: isolation and characterization of a new member of the family of human ETS transcription factors. Cell Growth Differ *3*, 705-713.
Weinstat-Saslow, D., Merino, M. J., Manrow, R. E., Lawrence, J. A., Bluth, R. F., Wittenbel, K. D., Simpson, J. F., Page, D. L., and Steeg, P. S. (1995). Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. Nat Med *1*, 1257-1260.
Westermarck, J., Seth, A., and Kahari, V. M. (1997). Differential regulation of interstitial collagenase (MMP-1) gene expression by ETS transcription factors. Oncogene *14*, 2651-2660.
Wheat, W., Fitzsimmons, D., Lennox, H., Krautkramer, S. R., Gentile, L. N., McIntosh, L. P., and Hagman, J. (1999). The highly conserved beta-hairpin of the paired DNA-binding domain is required for assembly of Pax-Ets ternary complexes. Mol Cell Biol *19*, 2231-2241.
Whittemore, A. S., Gong, G., and Itnyre, J. (1997). Prevalence and contribution of BRCA1 mutations in breast cancer and ovarian cancer: results from three U.S. population-based case-control studies of ovarian cancer. Am J Hum Genet *60*, 496-504.
Widschwendter, A., Tonko-Geymayer, S., Welte, T., Daxenbichler, G., Marth, C., and Doppler, W. (2002). Prognostic significance of signal transducer and activator of transcription 1 activation in breast cancer. Clin Cancer Res *8*, 3065-3074.
Wilkinson, D. A., Neale, G. A., Mao, S., Naeve, C. W., and Goorha, R. M. (1997). Elf-2, a rhombotin-2 binding ets transcription factor: discovery and potential role in T cell leukemia. Leukemia *11*, 86-96.
Wilson, C. A., Ramos, L., Villasenor, M. R., Anders, K. H., Press, M. F., Clarke, K., Karlan, B., Chen, J. J., Scully, R., Livingston, D., *et al.* (1999). Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas. Nat Genet *21*, 236-240.
Winqvist, R., Hampton, G. M., Mannermaa, A., Blanco, G., Alavaikko, M., Kiviniemi, H., Taskinen, P. J., Evans, G. A., Wright, F. A., Newsham, I., and et al. (1995). Loss of

heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. Cancer Res *55*, 2660-2664.
Wiseman, B. S., and Werb, Z. (2002). Stromal effects on mammary gland development and breast cancer. Science *296*, 1046-1049.
Wolvetang, E. J., Wilson, T. J., Sanij, E., Busciglio, J., Hatzistavrou, T., Seth, A., Hertzog, P. J., and Kola, I. (2003). ETS2 overexpression in transgenic models and in Down syndrome predisposes to apoptosis via the p53 pathway. Hum Mol Genet *12*, 247-255.
Wu, G. D., Lai, E. J., Huang, N., and Wen, X. (1997). Oct-1 and CCAAT/enhancer-binding protein (C/EBP) bind to overlapping elements within the interleukin-8 promoter. The role of Oct-1 as a transcriptional repressor. J Biol Chem *272*, 2396-2403.
Wu, K., Jiang, S. W., Thangaraju, M., Wu, G., and Couch, F. J. (2000). Induction of the BRCA2 promoter by nuclear factor-kappa B. J Biol Chem *275*, 35548-35556.
Xin, J. H., Cowie, A., Lachance, P., and Hassell, J. A. (1992). Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryonic cells. Genes Dev *6*, 481-496.
Xing, X., Wang, S. C., Xia, W., Zou, Y., Shao, R., Kwong, K. Y., Yu, Z., Zhang, S., Miller, S., Huang, L., and Hung, M. C. (2000). The ets protein PEA3 suppresses HER-2/neu overexpression and inhibits tumorigenesis. Nat Med *6*, 189-195.
Xu, C. F., Brown, M. A., Chambers, J. A., Griffiths, B., Nicolai, H., and Solomon, E. (1995). Distinct transcription start sites generate two forms of BRCA1 mRNA. Hum Mol Genet *4*, 2259-2264.
Xu, C. F., Brown, M. A., Nicolai, H., Chambers, J. A., Griffiths, B. L., and Solomon, E. (1997a). Isolation and characterisation of the NBR2 gene which lies head to head with the human BRCA1 gene. Hum Mol Genet *6*, 1057-1062.
Xu, C. F., Chambers, J. A., and Solomon, E. (1997b). Complex regulation of the BRCA1 gene. J Biol Chem *272*, 20994-20997.
Yamada, N., Tamai, Y., Miyamoto, H., and Nozaki, M. (2000). Cloning and expression of the mouse Pse gene encoding a novel Ets family member. Gene *241*, 267-274.
Yamamoto, H., Flannery, M. L., Kupriyanov, S., Pearce, J., McKercher, S. R., Henkel, G. W., Maki, R. A., Werb, Z., and Oshima, R. G. (1998). Defective trophoblast function in mice with a targeted mutation of Ets2. Genes Dev *12*, 1315-1326.
Yamashita, H., and Iwase, H. (2002). The role of Stat5 in estrogen receptor-positive breast cancer. Breast Cancer *9*, 312-318.
Yamashita, H., Iwase, H., Toyama, T., and Fujii, Y. (2003). Naturally occurring dominant-negative Stat5 suppresses transcriptional activity of estrogen receptors and induces apoptosis in T47D breast cancer cells. Oncogene *22*, 1638-1652.
Yang, S. H., Bumpass, D. C., Perkins, N. D., and Sharrocks, A. D. (2002). The ETS domain transcription factor Elk-1 contains a novel class of repression domain. Mol Cell Biol *22*, 5036-5046.
Yang, X., and Gabuzda, D. (1999). Regulation of human immunodeficiency virus type 1 infectivity by the ERK mitogen-activated protein kinase signaling pathway. J Virol *73*, 3460-3466.
Yordy, J. S., and Muise-Helmericks, R. C. (2000). Signal transduction and the Ets family of transcription factors. Oncogene *19*, 6503-6513.

Zhang, F., Li, C., Halfter, H., and Liu, J. (2003). Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. Oncogene *22*, 894-905.
Zhang, J., Johnston, G., Stebler, B., and Keller, E. T. (2001). Hydrogen peroxide activates NFkappaB and the interleukin-6 promoter through NFkappaB-inducing kinase. Antioxid Redox Signal *3*, 493-504.
Zhang, L., Eddy, A., Teng, Y. T., Fritzler, M., Kluppel, M., Melet, F., and Bernstein, A. (1995a). An immunological renal disease in transgenic mice that overexpress Fli-1, a member of the ets family of transcription factor genes. Mol Cell Biol *15*, 6961-6970.
Zhang, X., Blenis, J., Li, H. C., Schindler, C., and Chen-Kiang, S. (1995b). Requirement of serine phosphorylation for formation of STAT-promoter complexes. Science *267*, 1990-1994.
Zhao, F. Q., Adachi, K., and Oka, T. (2002). Involvement of Oct-1 in transcriptional regulation of beta-casein gene expression in mouse mammary gland. Biochim Biophys Acta *1577*, 27-37.
Zhong, Z., Wen, Z., and Darnell, J. E., Jr. (1994). Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science *264*, 95-98.
Zhou, J., Ng, A. Y., Tymms, M. J., Jermiin, L. S., Seth, A. K., Thomas, R. S., and Kola, I. (1998). A novel transcription factor, ELF5, belongs to the ELF subfamily of ETS genes and maps to human chromosome 11p13-15, a region subject to LOH and rearrangement in human carcinoma cell lines. Oncogene *17*, 2719-2732.