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THE ROLE OF *EWS/FLI-1* FUSION GENE IN EWING'S SARCOMA

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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In memory of my father

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ABSTRACT

Ewing's sarcoma (ES) and primitive neuroectodermal tumour (PNET) are tumours with poor prognosis that usually occur in children and young adults. Almost all of these tumours have a reciprocal translocation of human chromosome 22 (at the *EWS* gene) and either human chromosome 11 or 21 (at the *FLI-1* or *ERG* genes). Those translocations result in the production of aberrant chimaeric proteins consisting of the DNA binding domain of the ETS family transcription factors, FLI-1 or ERG, and a gene of unknown function, *EWS*. These fusion proteins are believed to play a key role in the pathogenesis of ES and PNET by modulating transcription of specific genes associated with oncogenesis. Thus, the ability of these fusion proteins to transform fibroblasts and the ability of a specific engineered repressor to inhibit this transformation were investigated in this study. Furthermore, since no animal model currently exists for this disease, the ability of a murine *EWS/Fli-1* fusion gene to transform fibroblasts was compared with the human *EWS/FLI-1* gene.

In this study, both murine and human EWS/FLI-1 proteins could similarly transform NIH3T3 cells, forming colonies in soft agar assay, proliferating in low serum medium and forming tumours in *BALB/c nu/nu* mice. Proto-oncogenes such as *EAT-2*, *Manic Fringe*, *Stromelysin-1* and *c-myc* genes were also upregulated in these cells. To elucidate whether EWS/FLI-1 is a dominant factor in transformation, a specific potent transcriptional suppressor, Kruppel associated domain (*KRAB*) fused to the FLI-1 DNA binding domain, was engineered to encounter the effect of EWS/FLI-1 and to reverse the transformation phenotype. When this fusion protein was stably expressed in EWS/FLI-1 transformed NIH3T3 cells, it resulted in loss of the ability of EWS/FLI-1 transformed cell lines to grow in low serum medium and soft agar, and impairment of tumour formation in *BALB/c nu/nu* mice. This was associated with down-regulation of *c-myc* at the protein and mRNA level and down-regulation of downstream targets such as *EAT-2* and *Manic Fringe* mRNA transcripts. In addition, introduction of a *KRAB/FLI-1* fusion gene into a human Ewing's sarcoma cell line, SK-N-MC, resulted in a similar growth retardation *in vitro* and *in vivo*, and down-regulation of *c-myc*. Taken together, these findings suggest that the transformed

phenotype of Ewing's sarcoma cells can be reversed by using the sequence specific FLI-1 DNA binding domain to target a gene repressor domain. This approach provides potential avenues for the elucidation of the biological mechanisms of EWS/FLI-1 oncogenesis and the development of gene therapy.

One approach to further investigate the aetiology of this fusion gene in ES/PNET is the generation of a mouse model which mimics the human ES/PNET translocation by homologous recombination and gene knock-in technology. The data presented in this thesis indicate that human and murine EWS/FLI-1 transforming in a similar manner and therefore development of such a model is possible and appropriate. This animal model will be used to both further understand this disease *in vivo* and examine potential therapies. Moreover, the *EWS* gene is involved in a large number and a diverse range of other human tumours, although the normal function of this gene and the manner in which it contributes to cancer are unknown. Thus, development of an *EWS* gene knockout would greatly assist in the elucidation of the normal biological function of *EWS* gene in this project. Both knock-in and knockout targeting vectors were generated. Although initial targeting attempts were unsuccessful, the identification of successfully targeted clones is continuing.

Furthermore, a novel ETS transcription factor, *ER99*, was identified in this project. This gene was expressed only in normal human testis, and in a variety of human cancer cell lines, including Ewing's sarcoma. It was also expressed in EWS/FLI-1 transformed NIH3T3 cells (but not in control cells) showing that *ER99* can be upregulated by the EWS/FLI-1 fusion protein. Further investigation of the oncogenicity of this gene will determine whether it has a role in the aetiology of these cancers.

DECLARATION

I declare that the material contained in this thesis has not been accepted for the award of any other degree or diploma in any University or Institute. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made.

... ... [redacted] ... ...

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COMMON ABBREVIATIONS

bp	=	base pairs
kb	=	kilobase pairs
DNA	=	deoxyribonucleic acid
cDNA	=	complementary deoxyribonucleic acid
RNA	=	ribonucleic acid
RNase	=	ribonuclease
dNTP	=	deoxynucleotide triphosphate
ATP	=	adenosine triphosphate
CTP	=	cytidine triphosphate
GTP	=	guanosine triphosphate
TTP	=	thymidine triphosphate
mQH_2O	=	milliQ (reverse osmosis/ultrafiltrated) water
^0C	=	degrees centrigrade
M	=	molar
Ci	=	curies
mol	=	mole
Mr	=	molecular weight
cpm	=	counts per minute
g	=	gram
l	=	litre
µl	=	micro litre
λ	=	lambda
cm	=	centimeter
U	=	unit
PCR	=	polymerase chain reaction

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Working Papers:

(1) **Chan D**, Wilson T, Xu D, Cowdery H, Hertzog P, Kola I. (2001) Transformation induced by EWS/FLI-1 is suppressed by KRAB/FLI-1. (In preparation)

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(1) **Chan D**, Sanij E, Wilson T, Xu D, Kola I (1999) Murine EWS/Fli-1 fusion protein mimics the transformation event in Ewing's sarcoma. *11th Lorne Cancer Conference. Victoria, Australia. February 11-14th, 1999.* (Poster Presentation)

(2) **Chan D**, Wilson T. Xu D, Kola I (1999) Suppression of EWS/Fli-1 activated gene expression by KRAB/Fli-1: a possible way for inhibition of tumour development in Ewing's sarcoma. *Third PeterMac Symposium: Initiation & Progression of Cancer. Melbourne, Australia. November 7-10th, 1999.* (Poster Presentation) **(The Best Poster Prize Award)**

(3) **Chan D**, Wilson T, Xu D, Kola I (2000) Suppression of EWS/Fli-1 activated gene expression by KRAB/Fli-1: a possible way for inhibition of tumour development in Ewing's sarcoma. *12th Lorne Cancer Conference. Victoria, Australia. February 10-13th, 2000.* (Poster Presentation)

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(5) Wilson T, **Chan D**, Xu D, Kola I, Hertzog P (2000) The role of EWS/Fli-1 fusion gene in Ewing's sarcoma. *2000 Hanson Symposium: Biology and Novel Therapeutics. November 13-16th, 2000.* (Oral and Poster Presentation)

Chapter 1

Introduction and Literature Review

1.1 Ewing Family of Tumours

1.1-1 Epidemiology and Aetiology

The Ewing family of tumours (ESFT) is a subgroup of small-round-cell tumours which have a common neural histogenesis and tumour genetics. Histologically, these tumour cells have similar features such as uniform, densely packed, small round cells with round nucleoli-free nuclei and indistinct cytoplasm (Llombart-Bosch *et al*, 1990; Sanders *et al*, 1997). Molecularly, these tumour are defined by specific chimaeric transcripts (Delattre *et al*, 1994). This family includes Ewing sarcoma of bone, extraosseous or soft tissue Ewing's sarcoma (ES), Askin tumours of chest wall, and peripheral primitive neuroectodermal tumours (PNET) of bone or tissues. This family was firstly described as an endothelioma by James Ewing (Ewing J, 1984).

ESFT is the second most common malignant bone tumour of children and adolescents. Nearly 70% of patients are under the age of 20, with a slight male predominance and an annual incidence in the US of 2.7 cases per million children (Gurney *et al*, 1996). These tumours occur mostly in whites or persons of Hispanic origin and are extremely rare in black and Asian children (Li *et al*, 1980, Fraumeni *et al*, 1981). The reason for this racial dichotomy is unknown. A recent report found that the size of intron 6 of the EWS gene differs between white and black people. This intron indicates the most common translocation breakpoint region in ES/PNET. In addition, sequence analysis showed genome plasticity mediated by *Alu* retroposition and recombination in this intron (Zucman *et al*, 1997). These data may give some insight into the racial difference of this cancer incidence.

The precise aetiology of ESFT is unknown. There is no linkage between these tumours and either radiation exposure or any cancer family syndromes (Yamamota *et al*, 1960; Tucker *et al*, 1987). However, a recent investigation showed that the early region (E1A) of human adenovirus type 5 induces a specific human fusion transcript (*EWS/FLI-1*) that is characteristic of Ewing tumors in Hela cells and keratinocytes, as well as in human cell lines immortalized by adenoviruses (Sanchez-Prieto *et al*, 1999). This association between a single viral gene and a specific human fusion transcript may indicate a direct link to chromosome translocation which occurs in many human tumours. Nevertheless, this finding needs to be verified and its mechanism elucidated.

1.1-2 Pathology

Ewing's sarcoma and PNET belong to the group of neoplasms commonly referred to as small round cell tumours. The tumour cells are uniformly shaped and undifferentiated, with a very low mitotic rate given the rapid growth observed clinically (Turc-Carel, 1991). The individual cells in Ewing's sarcoma are round, moderate size, clear and frequently with quite scant cytoplasm and a round to oval nucleus. Small to moderate areas of necrosis may be present in the tumour, sometimes together with dense pyknotic cells indicating apoptosis. Calcification is seldom observed. Classical Ewing's sarcoma cells frequently contain large amounts of intracellular glycogen. However, the significance of this is unclear since up to 35% of Ewing's sarcoma do not contain detectable glycogen (Kissane *et al*, 1983).

Ewing's sarcoma has a high level of expression of the MIC2p (sup 30-32) antigen (Kovar *et al*, 1990; Ambros *et al*, 1991). The MIC2 protein is a ubiquitous cellular component and is useful in the differentiation of the Ewing family of tumours from some other small round cell tumours. However, it is also present in several unrelated types of tumours which limits its reliability as a diagnostic indicator (Kovar *et al*, 1990; Ambros *et al*, 1991; Fellinger e*t al*, 1991). Thus this ESFT is poorly defined since none of the phenotypic markers can fully discriminate them from other small-round-cell tumours.

1.1-3 Clinical Presentation

The most common presenting symptoms in Ewing's sarcoma or PNET patients are pain, swelling, or both, at the site of the primary tumour. Patients may also present with systemic signs and symptoms such as weight loss, fever and increased erythrocyte sedimentation rate (Table 1.1) (Holcombe, 1997). These systemic signs are more common in patients presenting with metastases (Ladanyi *et al*, 1995).

Ewing's sarcoma or PNET can develop in almost any bone in the body. The Ewing family of tumours most commonly found in the pelvic bones, femur, humerus and ribs. Table 1.2 presents the primary sites for over 300 patients treated in the first Intergroup Ewing's Sarcoma Study (IEES) (Kissane *et al*, 1983). Soft tissue masses are common in Ewing's sarcoma, although occasionally a patient may have a large amount of bone involved with minimal mass even on (Computed Tomography) CT scan or (Magnetic Resonance) MR imaging (Boyko *et al*, 1987). The soft tissue masses often occur near bones and may be difficult to differentiate from primary tumours.

The probability of developing metastases in Ewing's sarcoma or PNET patients varies from individuals, but averages about 25% (Green, 1985). Common sites of metastases include lung (~50%), bone (~25%), and bone marrow (~20%) (Pilepich *et al*, 1981). Metastases to other sites such as the central nervous system are rare and are only observed during late disease relapses.

1.1-4 Staging and Prognosis

No widely accepted staging system for Ewing's sarcoma exists. Disease status and prognosis for Ewing's sarcoma is based on imaging of the primary tumour and sites of likely metastases. Plain films and MR imaging of the primary sites are taken while chest radiographs and CT scan of the lung, bone scan, and bone marrow biopsy are taken for checking metastases (Fig.1.1). Laboratory studies also include a complete blood count, erythrocyte sedimentation rate (increased in up to 50% of patients), and baseline chemistries (Holcombe, 1997). Prognosis clearly varies with the presence or absence of metastases, and with tumour size and location.

Local pain	84%
Local swelling	63%
Fever	28%
Paraplegia	3%

Table 1.1 Symptoms at Diagnosis of Ewing's sarcoma of bone. (Adapted from Grier, 1997; *Pediatr Clin North Am.* 44:991-1004).

Primary Site	Percent
Pelvic	20
Ilium	12.5
Sacrum	3.3
Ischium	3.3
Pubis	1.7
Lower extremity	45.6
Femur	20.8
Fibula	12.2
Tibia	10.6
Feet	2.0
Upper extremity	12.9
Humerus	10.6
Forearm	2.0
Axial Skeleton/ribs	12.9
Face	2.3

Table 1.2 Distribution of Primary Sites for Patients with Ewing's sarcoma of bone. (Adapted from Grier, 1997; *Pediatr Clin North Am.* 44:991-1004).

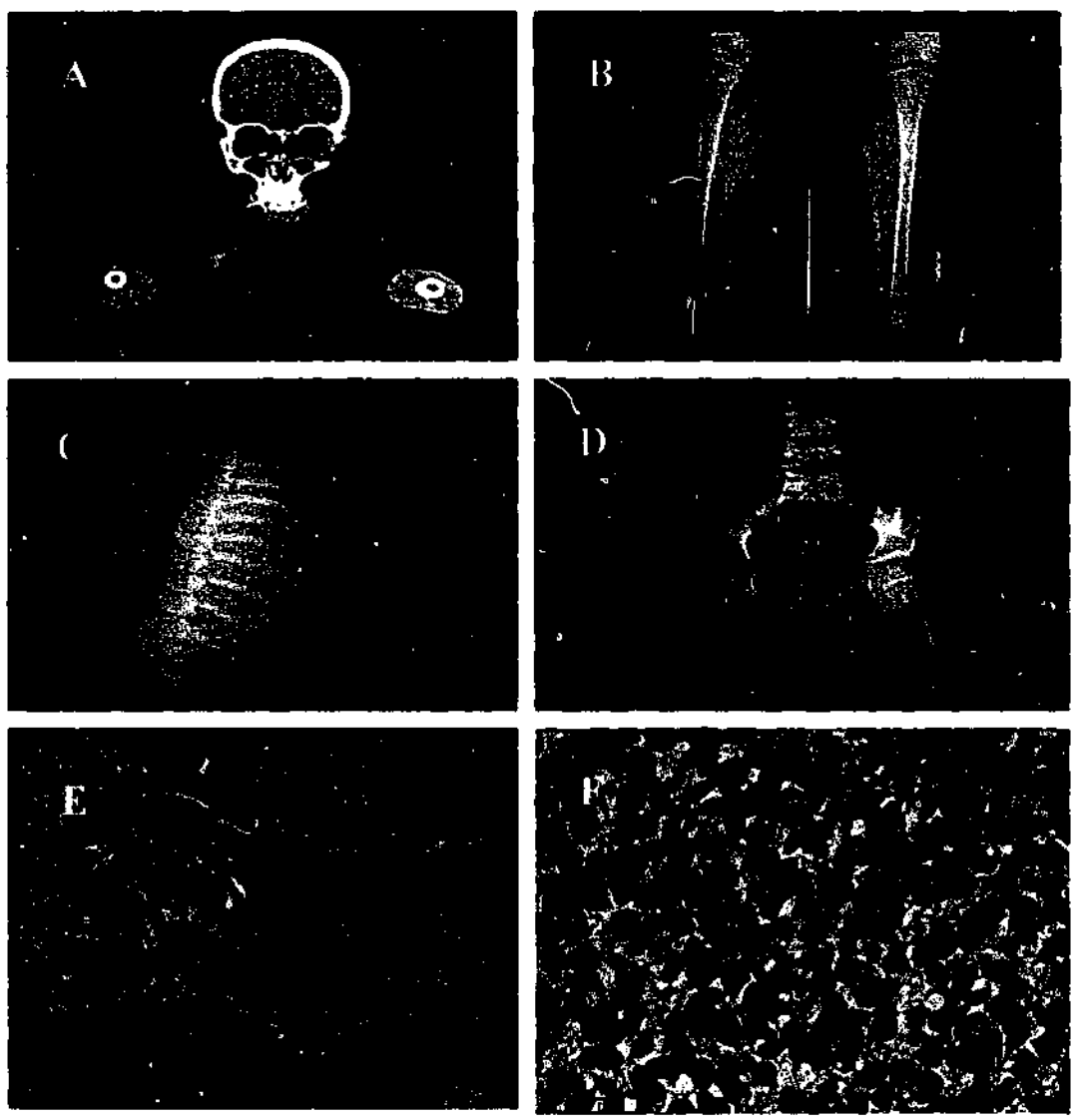


Fig. 1.1 CT scan images showing ES/PNET tumours exist in (A) skull, (B) fibula, (c) ribs and (D) cervical bone (adapted from website: http://korbl.sote/hu). (E-F) ES tumour cell morphology under light microscope (From website: http://med.univ-rennesl.fr)

1.1-5 Treatment

For the past two to three decades, tremendous progress has been made in the treatment of Ewing's sarcoma. With current therapy and supportive care, almost two thirds of children and adolescent patients with localized ESFT can expect to survive. These localized solid tumours arising from bones can be completely removed by surgery, radiotherapy, chemotherapy or a combination of therapy and surgery. The outcome of these treatments depends on the location of the primary site of localized tumour (Gehan *et al*, 1981). Surgery is the preferred approach if the lesion is amenable to surgery. Tumour control using radiotherapy requires moderately high doses. Standard practices require doses ranging from 5500 to 6000 cGy (Suit, 1975). Chemotherapy has improved local control rates in Ewing's sarcoma (Tepper *et al*, 1980). Among these approaches, surgery is the most effective way to treat the localized Ewing's sarcoma. However, the morbidity associated with surgically removing large tumours from important functional areas can be limiting this procedure. Therefore, surgery sometimes is used after the tumour volume is reduced by initial radiotherapy or chemotherapy.

Ewing's sarcoma is really a systemic disease with micrometastatic disease in place at the time of diagnosis. In metastatic Ewing's sarcoma/PNET, the tumour cells have spread most commonly to lung, bone, bone marrow, lymph node and central nervous system. The prognosis of this type of patients is poor. Systematic chemotherapy using multi-drugs such as vincristine, doxorubicin, cyclophosphamide, and actinomycin D (VAC/doxorubicin) has been standard for several decades. The addition of ifosfamide, with or without etoposide to the above regimen resulted in a significant improvement in event-free survival in ESFT (Grier *et al*, 1996). However, this addition has not appeared to improve survival rate in high-risk patients who have developed metastases (Miser *et al*, 1996; Wexler *et al*, 1996). This has led some clinicians to use megatherapy with stem-cell rescue in patients with metastatic Ewing's sarcoma at presentation or in those who develop metastases while on other therapies (Michon *et al*, 1994). However, the efficacy of megatherapy in Ewing's sarcoma is not yet clear.

Treatment after relapse is extremely difficult in Ewing's sarcoma. The minimal residual disease is difficult to monitor in ESFT. The identification of various prognostic features

that might help identify which patients might benefit from alternative therapies or be spared over-treatment will be important for effective treatment. Specific phenotypic markers for ESFT remain to be determined. The development of molecular prognostic indicators for ESFT is therefore of considerable importance.

1.2 Molecular Genetics in Ewing's sarcoma and Primitive Neuroectodermal Tumour

1.2-1 Biology of *EWS* and Related Fusion Genes in Ewing's Sarcoma (ES) and Primitive Neuroectodermal Tumour (PNET)

Many types of sarcomas are characterized by specific chromosomal translocations which are likely to be of aetiologic significance. Greater than 90% of cases of ES/PNET cells are characterized by a clonal translocation. Translocations can be visualized by standard cytogenetics in over 80% of the Ewing family of tumours, and can be further identified in over 90% cases by using molecular techniques (Downing *et al*, 1995; West *et al*, 1997). Among these translocations, approximately 85% involve rearrangement of chromosomes 11 and 22: t(11;22)(q24;q12) (Whang *et al*, 1986; Turc-Carel *et al*, 1986). Another 5%-10% involve an alternative rearrangement juxtaposing chromosomes 21 and 22: t(21;22)(q21;q12) (Zucman *et al*, 1993, Sorensen *et al*. 1994). There are some sporadic reports of other ES/PNET cytogenetic translocations involving the same region of chromosome 22 which was rearranged to other chromosomes such as chromosome 7: t(7;22)(q22;q12) (Jeon *et al*, 1995). Apart of these known reports, there are other rare translocation patterns involving chromosome 22 and other unidentified partners. Molecular cloning and characterization of these known and unknown translocation breakpoints (Delattre *et al*. 1992, Zucman *et al*, 1992) have provided new tools for unambiguous diagnosis of ES/PNET (Downing *et al*. 1993; Taylor *et al*. 1993; Toretsky *et al*. 1995) and opened new avenues for research into the biology of these tumours.

1.2-2 The t(11;22)(q24;q12) in ES/PNET

Molecular genetic characterization of ES/PNET translocations showed that most chromosome 22 breakpoints are clustered within a small 7 kb region of a novel gene, designated *EWS*, and chromosome 11 breakpoints are in a larger 40 kb region within the *FLI-1* gene (Zucman *et al*, 1992). The reciprocal translocation results in the production of a *EWS/FLI-1* transcript which joins the 5' end of the *EWS* gene on band q12 of chromosome 22 and the 3' end of the *FLI-1* gene on band q24 of chromosome 11 (Fig.1.2) (Delattre *et al*, 1992). This *EWS/FLI-1* fusion gene on der(22) chromosome is driven by an endogenous *EWS* promoter and is believed to encode an oncogenic chimaeric transcription factor (see Section 1.5) (Fig. 1.2) (Delattre *et al*, 1992; May *et al*, 1993; Bailly *et al*, 1994). On the other hand, the reciprocal *FLI-1/EWS* fusion on the der(11) does not express any transcript which has been shown by Northern blot hybridization and RT-PCR (Delattre *et al*, 1992; Bhagirath *et al*, 1995). The biological functions of *EWS* gene is still unknown, although the C-terminal of *EWS* appears to encode an RNA-binding domain and possesses RNA binding properties which has been demonstrated *in vitro* (see Section 1.3) (Delattre *et al*, 1992; Crozat *et al*, 1993; Ohno *et al*, 1994). The *EWS* gene is ubiquitously expressed. The *EWS* promoter region, which drives this constitutional expression, will regulate the expression of the chimaeric gene and is presumably responsible for the high-level expression of *EWS/FLI-1* in ES/PNET. The breakpoint on chromosome 11 involves the *FLI-1* gene, one member of the ETS transcription factor family with a sequence-specific DNA-binding domain at C-terminal. The translocation causes the RNA-binding domain of the *EWS* gene to be replaced by the DNA-binding domain of the *FLI-1* gene.

1.2-3 Molecular Diversity of *EWS/FLI-1* Fusion Transcripts

In ES/PNET, nine types of chimaeric *EWS/FLI-1* transcripts has been observed, representing different combinations of exons from *EWS* and *FLI-1* (Table 1.3) (May *et al*, 1993; Zucman *et al*, 1993; Delattre *et al*, 1994; Ladanyi *et al*, 1995). Among these *EWS/FLI-1* transcripts, type I translocation is the most predominant type followed by type II. Together, they account for about 80% of all cases with chimaeric *EWS/FLI-1*

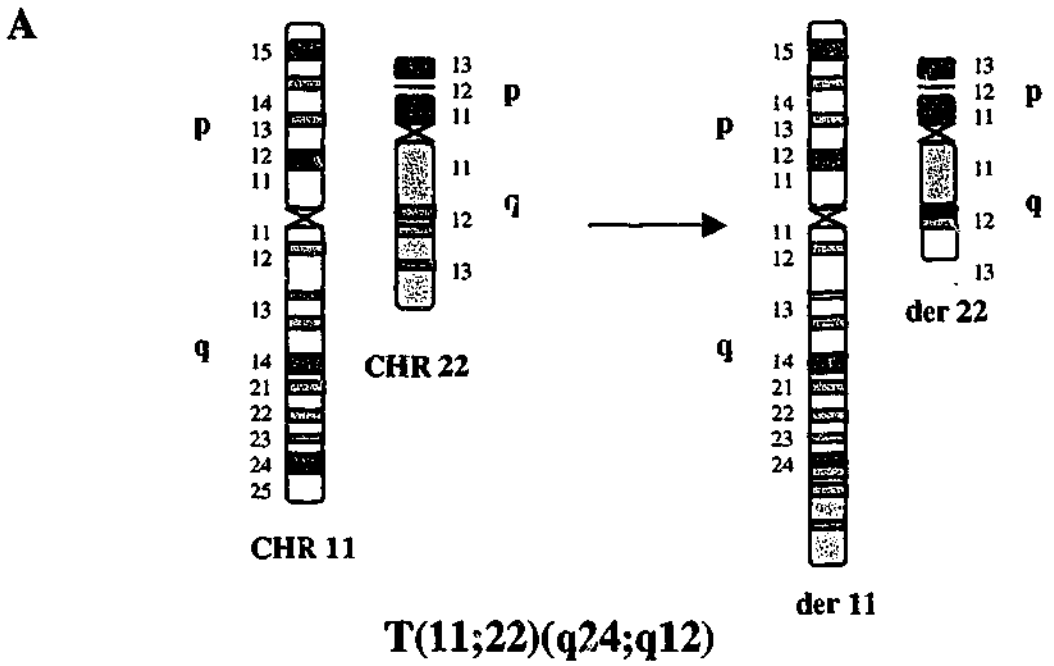


Fig. 1.2 (A) Schematic diagram of t(11;22)(q24;q12) chromosomal translocation associated with ES/PNET. (B) Schematic diagram of *EWS/FLI-1* fusion gene resulting from t(11;22). Individual gene exons are depicted as numbered boxes, and the line represents intervening and flanking DNA. The regions where chromosomal breakpoints occur are bracketed. (Adapted from de Alava *et al*, 2000; *J. Clin Oncol.* 18:204-213)

RNA transcripts (Ladanyi *et al*, 1995) (Table 1.3). These translocations fuse exon 7 of *EWS* to exon 6 of *FLI-1* and exon 7 of *EWS* to exon 5 of *FLI-1* respectively (Kim *et al*, 1999). The type II EWS/FLI-1 fusion protein has 22 additional amino acids insertion at the FLI-1 portion compared with the type I fusion protein (Fig.1.3) (Kim *et al*, 1999). Clinical investigations found that the type I *EWS/FLI-1* fusion in ES/PNET is associated with a better prognosis compared with other types of rearrangements. This was independent of tumor site, stage, and size (Zoubek *et al*, 1996; Alava *et al*, 1998). The prognosis is not due to decreased transactivation in reporter assays and reduced DNA binding affinity using electromobility shift assays of type I *EWS/FLI-1* compared with type II *EWS/FLI-1* fusion protein (Lin *et al*, 1999).

The essential structural elements of the chimaeric gene product, namely the entire DNA-binding domain of *FLI-1* and the entire transactivational N-terminal domain of *EWS*, are present in all *EWS/FLI-1* fusion transcripts. Restriction mapping of the breakpoints and analysis of the structure of the chimaeric RNAs indicate that approximately 90% of breakpoints happened within introns 7 and 8 of *EWS*, in approximately equal opportunities (Plougastel *et al*, 1993; Zucman *et al*, 1993). Interestingly, breakpoints in either of these introns result in *EWS/FLI-1* chimaeric transcripts that include exon 7 but not exon 8 of *EWS*, because exon 8 appears to be systematically spliced out in the chimaeric transcripts (Zucman *et al*, 1993). Other *EWS/FLI-1* rearrangements involve breakpoints within introns 9 and 10 of the *EWS* gene. The most predominant breakpoints within *FLI-1* involve introns 5 and 6 while others also are introns 4, 7 and 8 (Table 1.3) (Ladanyi *et al*, 1995).

In another 5% of ES/PNET, another t(21;22)(q22;q12) translocation is present instead of the t(11;22)(q24;q12). This translocation fuses *EWS* to another *ETS* family gene, *ERG*, which is highly homologous to *FLI-1* especially in the EDB domain, but located at 21q22 (Dunn *et al*, 1994; Sorensen *et al*, 1994). Unlike *FLI-1*, *ERG* is in the opposite orientation to *EWS* relative to the centromere (Dunn *et al*, 1994; Sorensen *et al*, 1994), and therefore a simple reciprocal exchange like t(11;22)(q24;q12) cannot explain the production of a functional fusion gene. Although the mechanism is not understood, the consequences of this rearrangement are thought to result in a similar spectrum of molecular variants (Zucman *et al*, 1993; Giovannini *et al*, 1994). Other rare variant ESFT translocations which have been observed are t(7;22)(p22;q12), where *EWS* is

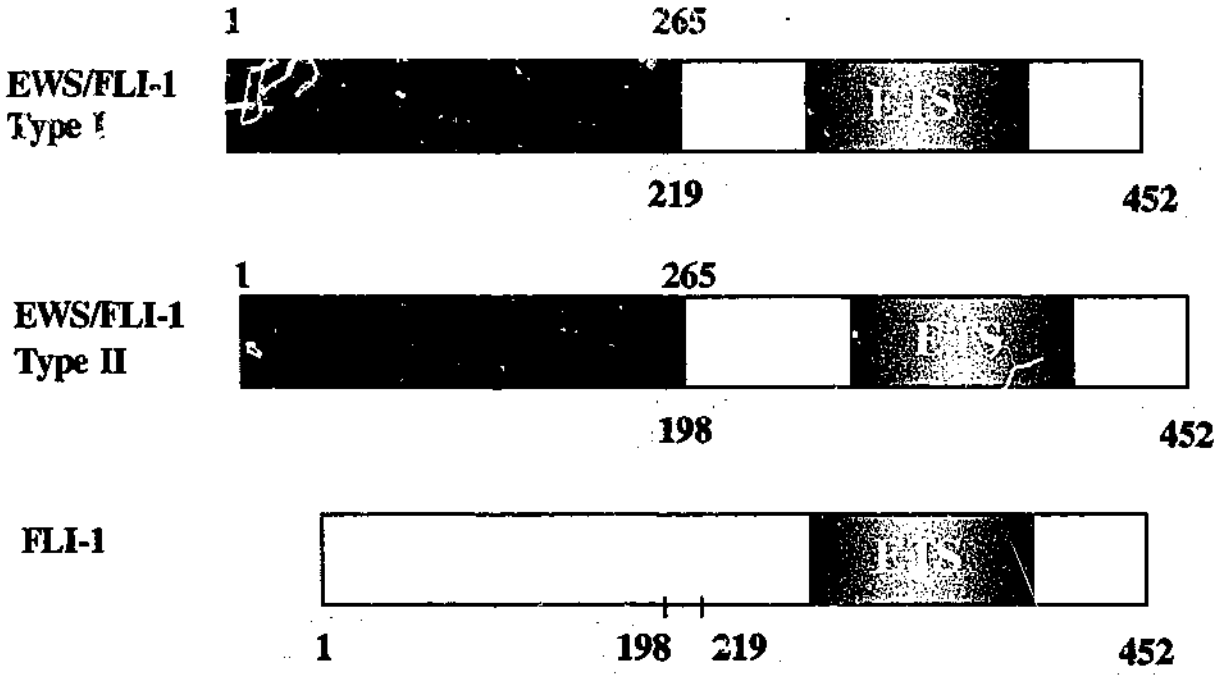


Fig. 1.3 Schematic Diagram showing of EWS/FLI-1 chimaeras generated by fusion of EWS and FLI-1. In both chimaeras, the carboxy-terminal portion of EWS containing the putative RNA-binding domain is replaced by the carboxy-terminal part of FLI-1 containing the ETS DNA-binding domain. The EWS N-Terminal Domain is shown as a blue-coloured box. The ETS domain is represented, and the differences between type I and type II EWS/FLI-1 fusion products are illustrated. The type II Ewing's sarcoma/FLI-1 protein contains the insertion of 22 additional amino acids from FLI-1. Positions of the interrupted codons are indicated in each case. (Adapted from Kim *et al*, 1999; *Physiol Genomics* 11:127-38).

Junction of			
EWS exon	to ***FLI*** exon	Frequency	Type
7	6	60-70%	I
7	5	20-25%	II
7	7	<1%	
7	8	2%	
9	4	2%	
9	7	2%	
10	5	6%	
10	6	4 – 5%	
10	8	2%	

Table 1.3 Combinatorial diversity of *EWS/FLI-1* fusion genes in ES/PNET (Adapted from Ladanyi, 1995; *Diagn Mol Pathol* 4:162-173)

fused to *ETV1* (Jeon *et al*, 1995); t(17;22)(q12;q12), where *EWS* is fused to *E1AF* (Urano *et al*, 1996); and t(2;22)(q33;q12), where *EWS* is fused to *FEV* (Peter *et al*, 1997). There are other unknown variants in which the precise genes involved have been defined such as t(1:16)(q11-25;q11-q24) (Ladanyi *et al*, 1995). In each case where the genes have been defined, the translocation which result in ES/PNET involves the 5' end of the *EWS* gene and an ETS family DNA binding domain.

1.2-4 Genomic Breakpoint Junction in t(11;22) Translocation in Ewing's sarcoma

Sequence analysis of these breakpoints found that the junctions were flanked by various oligomers, with a consensus sequence (5'-AGAAAARDRR-3'). Sequences highly homologous to *Alu* repeats and/or eukaryotic topoisomerase II cleavage sites (5'-RNYNNCNNGY$_{Y}$NGKTNYNY-3') (Spitzner *et al*, 1989), are also at or near located the breakpoints (Obata *et al*, 1999). These sequences may have a functional significance in the genesis of t(11;22) (Obata *et al*, 1999). Indeed, there are a number of examples of genetic diseases associated with *Alu* repeat mediated recombination, including sarcomas and hematological malignancies (Super *et al*, 1997; Panagopoulos *et al*, 1997). This hypothesis is consistent with a recent finding that *Alu* richness in *EWS* intron 6 could mediate the genome plasticity and explain the ethnic differences in the frequency of this translocation (Zucman *et al*, 1997).

There is also evidence of fragile sites in introns of *FLI-1*. Intron 4 of *FLI-1* has a high homology to *Sp* and *Sq* types of *La* sequences which are associated with common genomic breakpoints. However, there is none of *La* or other sequences were found in intron 7 (Obata *et al*, 1999). Breakpoints in *FLI-1* intron 4 occurred upstream to the *La* sequence (Obata et al, 1999). A consensus sequence for signals of breaking and fusion (5'-AGAAAARDRR-3') is found in both *EWS* and *FLI-1* introns where breakage occurs. Sequences homologous to the vertebrate topoisomerase II cleavage site (5'-RNYNNCNNGY$_{Y}$NGKTNYNY-3') is also near the intron 4 breakpoints of the *FLI-1* gene (Obata *et al*, 1999). These findings suggest that multiple mechanisms may

contribute to the breaking and rejoining of the chromosome 11 and 22 fragments in the genesis of t(11;22).

1.3 The Biology of the *EWS* Gene

1.3-1 Structure and Expression Pattern

The *EWS* gene maps to band q12 of human chromosome 22 and is oriented centromere to telomere. It contains by 17 exons spanning a 40 kb region. Sequence analysis of human *EWS* cDNA from a variety of cells revealed two alternatively spliced transcripts, *EWS* and *EWS-b* that differ with respect to the inclusion or exclusion of exon 8 and 9. (Fig. 1.4) (Ohno *et al*, 1994). Northern blot analysis revealed a heterogeneous band of *EWS* transcripts between 2.5-3.0 kb in a variety of human cells and normal tissues (Ohno *et al*, 1994; Aman *et al*, 1996). The half-life of *EWS* mRNA is approximately 120 min (Aman *et al*, 1996). The different lengths of poly-A^+ tails of each transcript that may give different stability or half-life for each transcript.

The full-length *EWS* transcript contains an open reading frame of 1968 bp which encodes a protein of 656 amino acids. This coding region can be divided into different domains (Fig. 1.4). The amino terminal region of *EWS* includes exons 1 to 7 (285 amino acids) and is rich in proline, glutamine, serine, and threonine and contains multiple copies of the hexapeptide repeat (Ser/Dly-Tyr-Ser/Gly-Gln-Gln/Ser-Ser/Gln/Pro) (Ohno *et al*, 1994). The region containing amino acids 257 to 262 from the amino terminal domain shares 40% homology with the C-terminal domain (CTD) of the large subunit of eukaryotic RNA polymerase II protein (CTD-pol II), which interacts with transcription factors to initiate the synthesis of mRNA. (Fig. 1.4) (Corden, 1990 and 1993).

The carboxyl terminal region of EWS contains an RNA-binding domain. Exons 11-13 encode a putative 85 amino acid RNA recognition motif (RRM) which is present in several RNA-binding proteins (Haynes, 1992; Kenan *et al*, 1991). There are three regions (exons 8-9, 14 and 16) that contain glycine-, arginine- and proline-rich (i.e

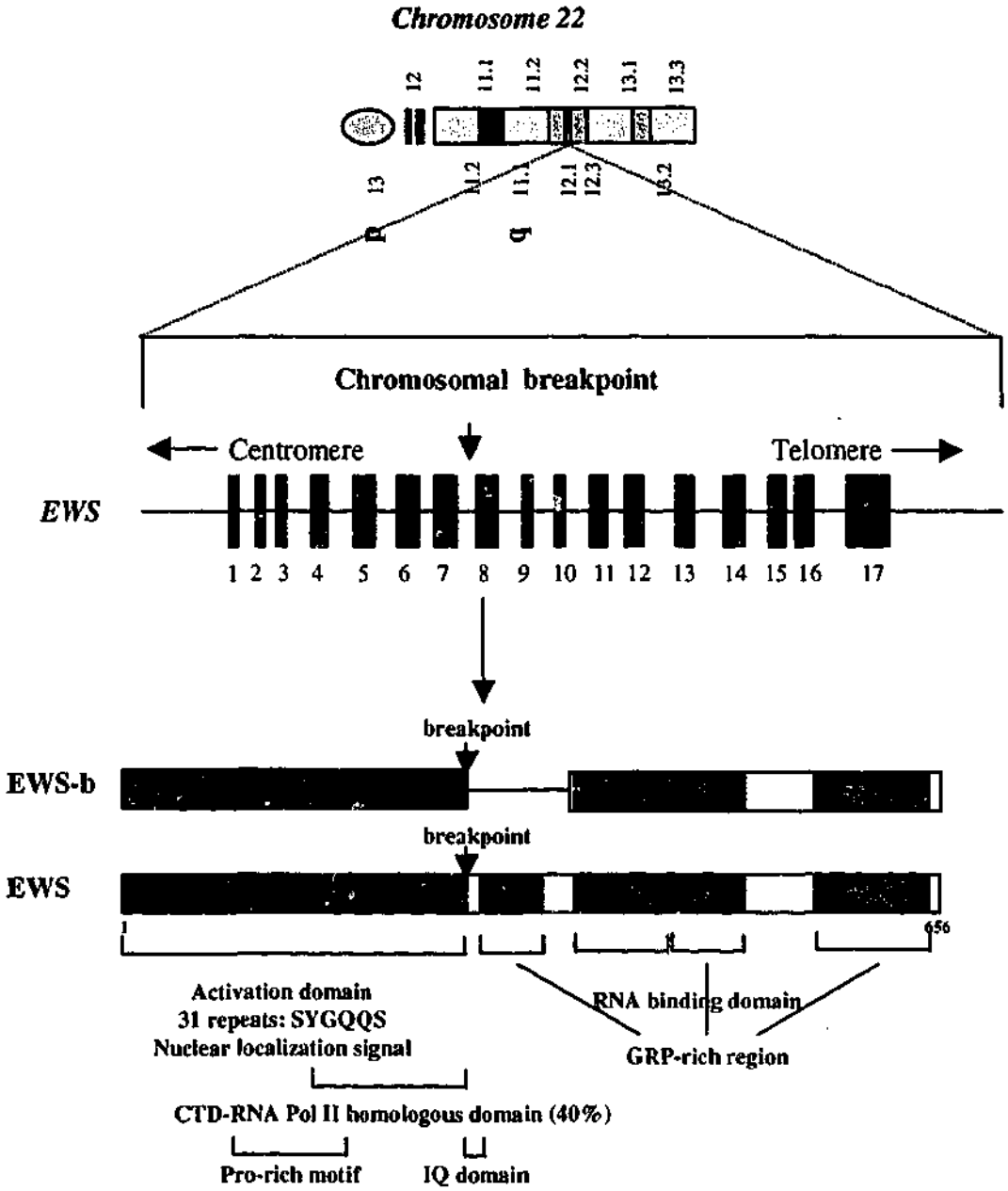


Fig. 1.4 Schematic representation of chromosomal localization and genomic organization of the *EWS* gene and the structural features of the EWS protooncoprotein. The *EWS* gene locates on chromosome 22q12 and contains 17 exons interrupted by 16 introns. The chromosomal breakpoint is indicated by an arrowhead. Restriction mapping of the rearrangements and analysis indicate that ~90% of breakpoints occur within introns 7 and 8, resulting in chimaeric RNAs that include exon 7 but not exon 8 of *EWS*. As a result of an alternative spilcing event that results in either inclusion or exclusion of exons 8 and 9, the EWS encodes two different isoforms: 1) EWS, which encodes a protein of 656 amino acids, and 2) EWS-b, which encodes a variant EWS polypeptide of 583 amino acids. The activation domain of EWS protein is represented by a shaded box. The three glycine-, arginine-, and poline-rich (GRP-rich) regions and RNA-binding domain are delineated. CTD, carboxy-terminal domian. (Adapted from Kim *et al*, 1999; *Physiol Genomics* 11:127-38).

GRP-rich) motifs (Fig. 1.4). They share high homology with a number of other proteins including various single-stranded nucleic acid-binding proteins, including SSB1 protein, nucleolin, fibrillarin, hnRNPs, and NOP1 (Kiledjian *et al*, 1992). This homology suggests that the carboxyl terminus of EWS is involved in RNA recognition or binding. Consistent with this interpretation, in vitro RNA binding activity assays have shown that EWS can bind to the ribonucleotide homopolymers, and that the conserved GRP-rich box present in the extreme carboxyl terminal region of EWS can function as an RNA-binding domain (Fig.1.4) (Ohno *et al*, 1994). Interestingly, the RNA-binding between the EWS and EWS-b isoforms is different: Compared with EWS, EWS-b can efficiently bind to RNA *in vitro* and especially to poly(G) and poly(U) (Ohno *et al*, 1994).

EWS contains an IQ domain (Fig. 1.4) that is phosporylated by protein kinase C (PKC) and interacts with calmodulin (CaM) (Deloulme *et al*, 1997). The IQ domain contains 20 amino acids that serve as a general regulatory domain in proteins. PKC phosphorylation of EWS inhibits its binding to RNA homopolymers. However, when CaM binds to EWS. It will inhibit PKC phosphorylation of EWS (Deloulme *et al*, 1997). Therefore, the IQ domain in EWS may coordinate the regulation of RNA processing by interacting with PKC and CaM.

The 5' region of the *EWS* gene has features of CpG-rich island and lacks canonical promoter elements, such as TATA and CCAAT consensus sequences (Plougastel *et al*, 1993). A high content of unmethylated CpG dinucleotides is a common feature of the promoter region for many housekeeping genes (Bird, 1986), suggesting that the *EWS* gene may have a housekeeping role which may explain its widespread expression (Zucman *et al*, 1993).

1.3-2 EWS belongs the Family of RNA Binding Proteins

Cellular proteins are regulated at multiple levels including transcription, mRNA stability and processing, translation, and protein stability and modification. RNA binding proteins are important components of regulation at the mRNA level (McKeown, 1992; Peltz *et al*, 1993). Several of these RNA binding proteins recognize

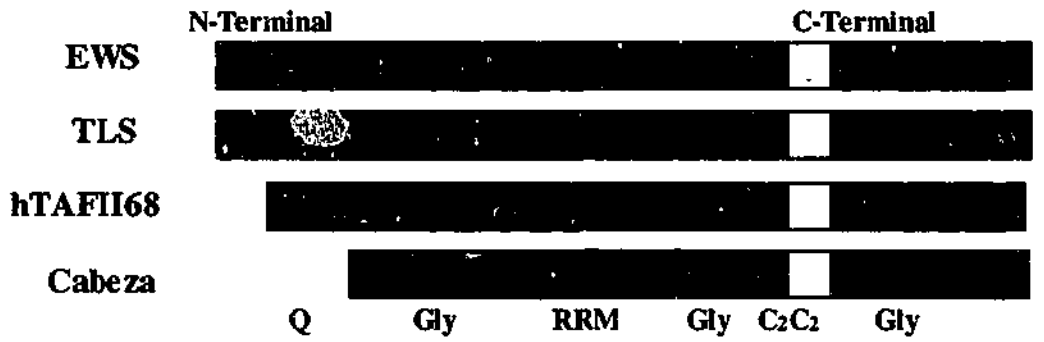
specific RNA sequences directly, while others recognize secondary and tertiary structural features of the RNA (Hedley *et al*, 1991; Tsai *et al*, 1991; Valcarcel *et al*, 1993; Saccomanno *et al*, 1994). A common motif shared by many RNA binding proteins is an 80-90 amino acid domain, often referred to as an RNA recognition motif (RRM) or RNA binding domain (RBD) (Birney *et al*, 1993; Burd *et al*, 1994). Within most RRMs are two highly conserved sub-domains known as RNP-1 and RNP-2 (or 2 RRP-rich regions see Section 13-1) (Fig.1.4, Fig.1.5) (Birnet *et al*, 1993; Burd *et al*, 1994; Kim *et al*, 1999). This motif within a protein usually indicates that it is capable of binding RNA and/or ssDNA. TLS/FUS, EWS, hTAF$_{II}$68 and Cabeza belong to a novel sub-family of the TET family of RNA-binding proteins that all contain and share this consensus RNA-binding domain RRP-rich regions (RNP-1 and RNP-2) (Fig. 1.5) (Delattre *et al*, 1992; Crozat *et al*, 1993; Stolow *et al*, 1995; Bertolotti *et al*, 1996). The RNP-1 domain of these proteins contains an acidic residue at the second position and a threonine in the fourth position, but lacks hydrophobic residues found in most other RNA-binding proteins (Bertolotti *et al*, 1996). Moreover, the RNP-1 and RNP-2 motifs of these proteins contain an abnormally long loop immediately after the first α helix (Bertolotti *et al*, 1996). This suggests that TET family members bind RNA and/or ssDNA in a unique way and differ from other members of TET family. Further, these TET sub-family proteins interact directly with several subunits (TAF$_{II}$s) of TFIID complexes by protein-protein interactions, suggesting that they are characterized not only by structural but also by functional homology (Bertolotti *et al*, 1996).

1.3-3 Translocation Partners of *EWS* in Other Tumour Types

Desmoplastic Small Round Cell Tumors (DSRCT)

DSRCT is a poorly understood malignant neoplasm in adolescents and young adults with widespread abdominal serosal involvement (Gerald *et al*, 1995). This tumour is associated with a specific chromosomal translocation, t(11;22)(p13;q12) (Table 1.4) that fuses the EWS amino terminus to the carboxyl terminus of WT1 (Fig. 1.6) (Biegel *et al*, 1993; Rodriguez *et al*, 1993; Shen *et al*, 1992; Gerald *et al*, 1995). The *WT1* gene

A)



B)

EWS(362) AIYVQGLNDSVTLDDLADFFKQCGVVKMNKRTGQPMI HIY LDKETGKPKGDATVSYEDPPTAKAAVEWFDGKDFQGSKLKVSLARKK

TLS (286) T I FVQGLGENVTI E SVADYFKQIG I I K TN KKTGQPMINLYTDRETGKLK GEATVSFDDPPSAKAAI DWFDGKE FSGNP IKVSFATRR

hTAF$_{II}$68 (232)T I F VQGLGEGVSTDQVGEFFKQ IG I I KT N KKTGKPMINLYTDKDTGKPK GEATVSFDDPPSAKAAI DWFDGKEFHGNI I KVSFATRR

Cabem (120) T I F VS GMDPSTTEQDI ETHFGA IG I I KKD KRTMKPKIWLYKNKETGASK GEATVTYDDTNAAQSAIEWFDGRDFNGNAIKVSLAQRQ

RNP2 RNP1

Fig. 1.5 Structural features of sub-family of TET family. (A) Structure comparison, (B) and amino acid sequence comparison in RRM (which consists of RNP1 and RNP2 motifs) among the members in this sub-family (Adapted from Stolow *et al*, 1995; *Nucleic Acids Res.* 23:835-843)

Tumour Type	Chromosomal Rearrangement	Genes at Chromosomal Breakpoints
Ewing's sarcoma	t(11;22)(q24;q12)	*EWS* (22q12), *FLI-1* (11q24)
	t(21;22)(q22;q12)	*EWS* (22q12), *ERG* (21q22)
	t(7;22)(p22;q12)	*EWS* (22q12), *ETV1* (7p22)
	t(17;22)(q21;q12)	*EWS* (22q12), *E1A-F* (17q21)
	t(2;22)(q33;q12)	*EWS* (22q12), *FEV* (2q33)
Desmoplastic small round cell tumour	t(11;22)(p13;q12)	EWS (22q12), *WT1* (11p13)
Melanoma of soft parts	t(12;22)(q13;q12)	*EWS* (22q12), *ATF1* (12q13)
Myxoid liposarcoma	t(12;22)(q13;q12)	*EWS* (22q12), *CHOP* (12q13)
	t(12;16)(q13;p11)	*TLS/FUS* (16p11), *CHOP* (12q13)
Human extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	*EWS* (22q12), *TEC(CHN)* (9q22)
Myeloid leukaemia	t(16;21)(p11;q22)	*TLS/FUS* (16p11), *ERG* (21q22)

Table 1.4 Tumour-specific chromosomal translocations involving *EWS* or related family members. (Adapted from Kim *et al*, 1999; *Physiol Genomics* 11:127-38).

spans ~50 kb and contains 10 exons (Fig. 1.6) and encodes a ~50 kDa transcription factor that has been shown to function as a tumour suppressor gene (Call *et al*, 1990). The last four exons encode individual zinc fingers of the Kruppel C_2-H_2 class. Alternative splicing of the *WT1* gene creates two isoforms, the major isoform (KTS+) and the minor isoform (KTS-), by removing or adding three amino acids between zinc fingers III and IV. These isoforms have different DNA-binding specificities, which probably causes different physiological effects (Rauscher *et al*, 1990; Bickmore *et al*, 1992). The *EWS/WT1* fusion transcript also produces two isoforms retaining the alternative splicing events between zinc fingers III and IV (Fig. 1.6) (Gerald *et al*, 1995; Rauscher *et al*, 1990). Introduction of *EWS/WT1* (-KTS) into NIH3T3 fibroblast cells results in increased growth rate, gain of anchorage-independent growth and tumour formation in nude mice. In contrast, the *EWS/WT1* (+KTS) isoform showed no transforming potential in these cells (Kim *et al*, 1998). It is thought that EWS/WT1 (-KTS) might transform by deregulating or activating a wider range of WT1 downstream targets. Some of these downstream targets have been shown to be activated by EWS/WT1 (-KTS) fusion protein, for example, *bcl-2, c-myc, EGFR* (*epidermal growth factor receptor*), *IGF-IR* (*insulin-like growth factor-I receptor*), *PDGF-A* (*platelet derived growth factor*), *IGF-II, CSF-1* (*colony-stimulating factor*), *TGFβ1* (*transforming growth factor*), *EGR-1* (*early growth response-1*), *Pax-2, Pax-8, c-myb, G-protein αi-2, Ki-ras, insulin receptor, p21, Nov-H, RAE-a, Inhibin-a, syndecan-1, midkine, Dax-1, and WT1* (Kim *et al*, 1998; Lee *et al*, 1997). Some of these genes have oncogenic potential such as autocrine and paracrine growth factors. Hence, they may explain the oncogenic properties of *EWS/WT1* (-KTS) chimaeric protein.

Malignant Melanoma of Soft Parts (MMSP)

MMSP is a rare tumour with a balanced chromosomal translocation, t(12;22)(q13;q12), which results a protein fusion between the amino-terminal 325 amino acids of EWS and the carboxyl-terminal 206 amino acids of ATF1 (activation transcription factor 1) (Table 1.4) (Zucman *et al*, 1993). ATF1 belongs to a member of bZIP transcription factors family that includes the cAMP response element binding protein (CREB) and activator forms of the cAMP response element modulator (CREM) (Lee *et al*, 1993). By fusing the N-terminal of EWS to C-terminal of ATF1, the transactivation ability of

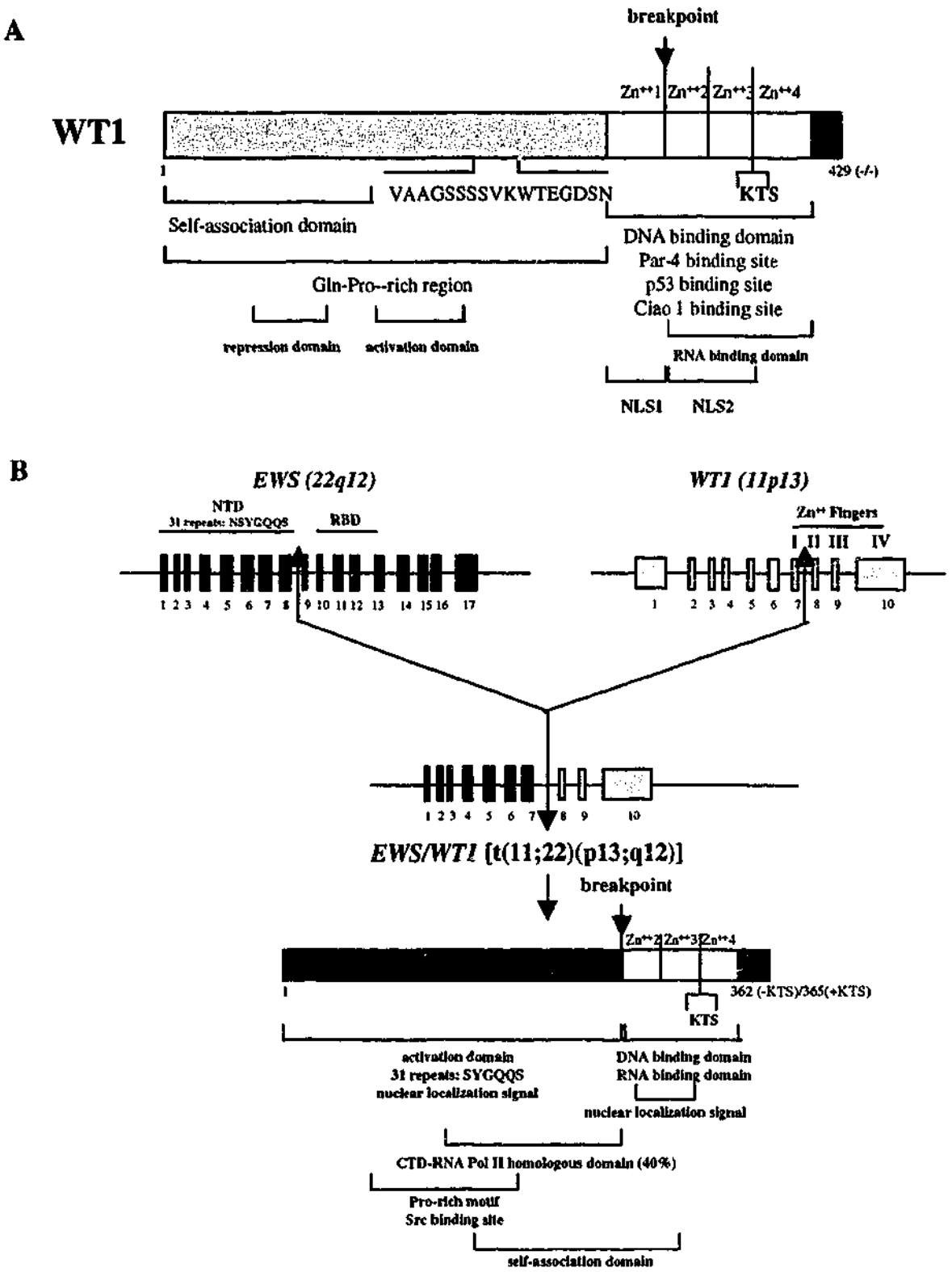


Fig.1.6 (A) Schematic diagram of the WT1 protein showing various functional domains. (B) Schematic diagram of the t(11;22)(p13;q12) of desmoplastic small round cell tumour (DSRCT) presenting the structure of normal *EWS* and *WT1* genes, the *EWS/WT1* fusion gene resulting from the chromosomal translocation, and the chimaeric EWS/WT1 protein. Exon and intron structure of the *EWS*, *WT1*, and *EWS/WT1* genes are shown with blue and orange boxes. As a result of alternative splicing event involves a splicing acceptor site in exon 9, causing the presence or absence of a 3-amino acid insertion (±KTS) between zinc fingers 3 and 4. (Adapted from Kim *et al*, 1999; *Physiol Genomics* 11:127-38).

ATF1 becomes stronger. Hence, in contrast with ATF1, EWS/ATF1 functions as a potent constitutive activator of several cAMP-inducible promoters such as *somatostatin*, *c-fos*, and *vasoactive intestinal polypeptide* (VIP) (Brown *et al*, 1995; Fujimura *et al*, 1996). As EWS/ATF1 has high potential to upregulate cAMP-inducible promoters, many of its downstream targets are dysregulated and induce the transformed phenotypes in MMSP cells.

Myxoid Liposarcoma

Some of myxoid liposarcoma is associated with a t(12;22)(q13;q12) translocation which results a chimaeric fusion gene, *EWS/CHOP*, while others are associated with a t(12;16)(q13;p11) translocation which results in another fusion gene, *TLS/FUS-CHOP* (Table 1.4). These translocations result in the RNA-binding domain at the 3' end of *EWS* or *TLS/FUS* being replaced by the basic leucine zipper domain of *CHOP* (Ron and Habener, 1992; Crozat *et al*, 1993). *TLS* (translocated in lymphosarcoma), also termed *FUS* (fusion), has extensive sequence similarity (55.6% identity) to the *EWS* gene (Crozat *et al*, 1993) and both belong to the sub-family of TET RNA binding proteins (see Section 1.3-2). The *CHOP* gene (*C/EBP homologous protein 10*) is a growth arrest and DNA-damage inducible member of the C/EBP family of transcription factor (Ron *et al*, 1992). CHOP is implicated in adipocyte differentiation and growth arrest (Butterwith, 1994; Ron *et al*, 1992). It is possible that the EWS/CHOP fusion product interferes with the normal transcription functions of C/EBP and causes a blockade in adipocyte differentiation which may explain the tumourigenecity of myxoid liposarcoma.

Human Extraskeletal Myxoid Chondrosarcoma

Approximately 75% of the myxoid variant of chondrosarcoma has been found to have a specific chromosomal translocation t(9;22)(q22;q12) which fuses the N-terminal domain of the *EWS* gene to the entire coding region of *TEC* gene (translocated in extraskeleton myxoid chondrosarcoma, also known as *CHN*) (Table 1.4) (Clark *et al*, 1996; Labelle *et al*, 1995). Although the precise fusion of TEC(CHN) is unknown, it shows sequence homology to the Nur-related factor 1 family of orphan nuclear receptors that are involved in the control of cell proliferation and differentiation by

modulating the response to growth factors and retinoic acid (Hibshoosh *et al*, 1997). The EWS/TEC(CHN) fusion protein has been shown to be a potent transcriptional activator compared with TEC(CHN) (Labelle *et al*, 1999). It may dysregulate TEC(CHN) downstream targets similar to EWS/FLI-1, EWS/WT1, or EWS/ATF1 etc. and result in transformation.

1.4 The ETS Family of Transcription Factor

1.4-1 ETS Structure and Functional Domains

As mentioned above, the aetiology of ES/PNET involves the expression of a fusion protein consisting of EWS and an ETS DNA binding domain (EDB domain). The ETS family of proteins is composed of an increasing number of transcription factors which are involved in a variety of developmental processes and cellular responses to external stimuli. The first ETS factor, the *ETS* oncogene v-*ets,* was discovered as part of a fusion protein with *gag* and *myb* expressed by the E26 avian erythroblastosis virus (LePrince *et al*, 1983; Nunn *et al*, 1983). Subsequently, a series of *ETS* genes was identified (Fig. 1.7) (Graves et al, 1998) and these proteins are found throughout the metazoan world, including the human, mouse, chicken, Xenopus, sea urchin and Drosophila (Macleod *et al*, 1992; Wasylyk *et al*, 1993). They are characterized by a highly conserved ETS DNA binding domain (EDB domain) of ~ 85 amino acid which can specifically binds to a 10 bp motif with the core consensus sequence C/A GGA A/T (Table 1.5) (Papas *et al*, 1987; Papas *et al*, 1989; Fisher *et al*, 1991; Seth *et al*, 1992; Nye *et al*, 1992).

1.4-1.1 ETS DNA Binding Domain (EDB Domain)

Interaction with a specific DNA sequence is a hallmark of transcription factors. This function is achieved by a part of the protein termed the ETS DNA Binding (EDB) Domain. This domain contains a conserved stretch of ~85 amino acids which can specifically bind to the motif C/A GGA A/T in the middle of 10 bp of DNA (Table 1.5) (Nye *et al*, 1992; Sharrocks *et al*, 1997). ETS proteins can be divided into sub-families, based on sequence similarity in the ETS domain (Fig.1.7), the relative position of the ETS domain in the protein, and additional similar sequences found only in sub-families.

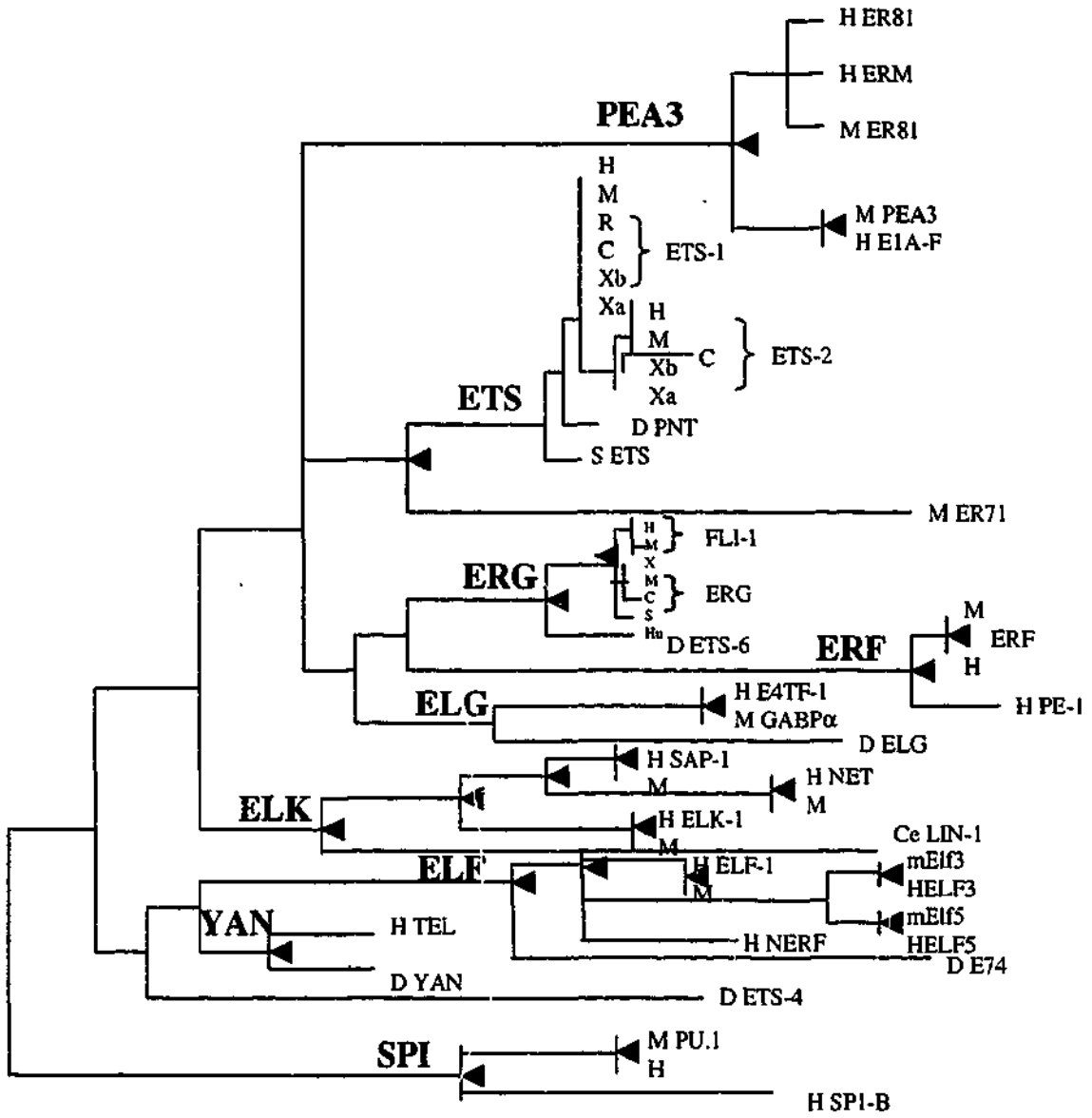


Fig. 1.7 Rooted phylogenetic tree of ETS family of transcription factors relating to their ETS domain sequences. (Adapted from Graves *et al*, 1998; *Adv Cancer Res.* 75:1-55)

GROUP	PROTEIN	SELECTED CONSENSUS			REFERENCE
ELF	**D-E74**	NNNNN AACCA T C	NNN GGA	NNN AGT	(Urness and Thummel, 1990)
	M-Elf-1	NNNNN AACCA t a	NNN GGA	NNNN AGTa g c	(John *et al*, 1996)
ELG	**M-GABPα**	NNN GCC a g a	NNN GGA	NNNN AGTN t a c	(Brown and McKnight, 1992)
ELK	**M-SAP-1**	NNN ACC	NNN GGA	NNN AGT t a c	(Shore and Sharrocks, 1995)
	M-Elk-1	NNN ACC	NNN GGA	NNNN AGTG A	(Shore and Sharrocks, 1995)
ERG	**M-Fli-1**	NNNNNN TNGACC g A	NNN GGA	NNNN AGTA a cG	(Mao *et al*, 1994)
	M-ER71	NNN GCC CG a	NNN GGA	NNNN TGTC Aac	(Brown and McKnight, 1992)
ETS	**M-Ets-1**	NNN ACC g a	NNN GGA	NNNN AGCN Ta T	(Nye *et al*, 1992)
PEA3	**M-ER81**	NNN GGC a c a	NNN GGA	NNNN TGTN T a c	(Brown and McKnight, 1992)
SP1	**M-PU.1**	NNNNNNN AAAAAGA t cC G	NNN GGA	NNNNN AGTAG c Gc	(Ray-Gallet *et al*, 1995)
	M-Spi-B	NNNNNNN AAAAAGA t c c	NNN GGA	NNNNN AGTAN c T	(Ray-Gallet *et al*, 1995)

Table 1.5 Selected DNA consensus binding sequences for ETS proteins. Consensus sequences were selected using DNA duplexes which randomized sequences. Nucleotide positions randomized (N) or fixed (G or A) during the *in vitro* selections are indicated above each sequence. Repeated selections were used in all cases. The highly conserved GGA core motif is boxed. Nucleotides in lowercase letters were less frequently selected. (From Graves *et al*, 1998; *Adv Cancer Res.* 75:1-55)

ETS proteins, except GABPα (which binds to ETS sites only as a heterodimer with the ankyrin repeat containing protein GABPβ), bind as monomers to DNA sequences containing a purine-rich core, C/A GGA A/T (Karim *et al*, 1990; Jannknecht *et al*, 1992; Nye *et al*, 1992; Woods *et al*, 1992). The adjacent flanking sequences on both sides of the C/A GGA A/T core determine the affinity of specific ETS proteins to these target sequences (Table 1.5). However, there is a variety of different ETS proteins that can bind with comparable affinities to the same target sequences *in vitro* (Brown *et al*, 1992) (Dr. R Thomas, personal communication). This suggests that several ETS proteins may contribute to the regulation of each target gene *in vivo* and that the precise regulation by these ETS factors depends upon not only their affinity of DNA binding, but also on protein-protein interactions (see Section 1.4-2).

Although the ETS-domain is sufficient for direct DNA-binding, protein regions outside the EDB domain have been shown to modulate this activity in ETS-1, Elk-1 and SAP-1. In ETS-1, three such regions seem to negatively influence DNA-binding: a central region of ~70 amino acids (Lim *et al*, 1992), a 90 amino acids long stretch lying upstream of the EDB domain (Wasylyk *et al*, 1992), and the carboxyl-terminus (Lim *et al*, 1992; Hagman *et al*, 1992). It has been proposed that the 90 amino acid region upstream of the ETS-domain is capable of binding to and masking the DNA-binding surface (Wasylyk *et al*, 1992). The other repressor domains are believed to bind each other, masking the intervening EDB domain and preventing it from binding DNA (Lim *et al*, 1992). However, these regions are not present in all ETS proteins. For example, deletion of PU.1 sequences either upstream or downstream relative to the EDB domain did not result in enhanced DNA-binding (Hagman *et al*, 1992; Wasylyk *et al*, 1992).

1.4-1.2 Transactivation Domains

The transactivation domains of transcription factors mediate direct or indirect interaction with the basal transcriptional machinery, resulting in enhanced transcription (Mitchell *et al*, 1989; Tjian *et al*, 1994; Goodrich *et al*, 1996; Ranish *et al*, 1996). Unlike the EDB domain, the transactivation domains are not highly conserved between

different ETS transcription factors and their interactions with other proteins are not well known. These regions are characterized by 30 to 100 amino acid stretches rich in acidic (Bohmann *et al*, 1987; Roberts *et al*, 1993; Tjian *et al*, 1994), glutamine (Courey *et al*, 1988; Mitchell *et al*, 1989; Tjian *et al*, 1994) or proline residues (Williams *et al*, 1988; Ko *et al*, 1988). These regions are found in the amino terminus of ETS-1, ETS-2, ERG, FLI-1 and GABPα and have been found in the Drosophila ETS-like protein Yan/Pok (Lai *et al*, 1992; Tei *et al*, 1992). Moreover, these transcriptional activation domains have a helix-loop-helix motif similar to c-Myc, MyoD and E12 that may be involved in protein-protein interactions (Seth *et al*, 1990). The variability in this region between ETS family members would affect their ability to interact with other transcriptional components and thus may have implications for the specificity of transactivation.

1.4-1.3 Nuclear Localization Domains

Most transcription factors contain a nuclear localization signal (NLS) which directs them to the nucleus. Intracellular localization is an important means of regulation of transcription factor activity. This regulation may involve retention in the cytoplasm by binding to anchoring proteins, masking of the NLS, transcription factor phosphorylation and/or modulation of nuclear import (Gerace, 1992; Jans, 1995; Vandromme *et al*, 1996). The mechanism by which such tags specify nuclear localization appears to initially involve nuclear pore receptor molecules such as importin (Gerace, 1992; Gorlich *et al*, 1995; Vandromme *et al*, 1996). Subsequently, proteins are actively transported inside the nucleus by ATP/GTP dependent mechanisms (Newmeyer *et al*, 1988; Jans, 1995; Corbett *et al*, 1996). In ETS transcription factors, the NLS is located near the EDB domain and characterized by a peptide tag (GKRKNKPK) (Boulukos *et al*, 1989). For example, *ETS-2* has been described as a nuclear oncogene in several cancers (Sapi *et al*, 1998; Sementchenko *et al*, 1998; Foos *et al*, 1998; Foos and Hauser, 2000). In contrast to cancer cell lines, the majority of ETS-2 is localized in the cytosol in G_0/G_1 and in the nucleus of M-phase primary embryonic fibroblasts. Under oxidative and/or genotoxic stress, ETS-2 protein is rapidly translocated from the cytosol into the nucleus. This is the first example of an ETS-transcription factor undergoing nuclear-cytoplasmic shuttling (Dr. E. Wolvetang, personal communication).

1.4-2 ETS Transcription Factors in Gene Regulation

Gene transcription is central to all cellular processes. From the time of embryonic genome activation onward, genetic instructions are expressed. This occurs through mechanisms involving transcription of genes in mRNA, and then translation of mRNA into functional and structural proteins. Among these events, gene transcription is perhaps the most important regulatory aspects. All ETS transcription factors bind to unique GGAA/T DNA sequence (EDB domain) and belongs to a big transcription family. Such EBS have been identified in the promoter/enhancer regions of viral and cellular genes. Thus, the proper expression control of downstream target genes by ETS factors will be important in the regulation of cellular proliferation, differentiation, development, haematopoiesis, apoptosis, metastasis, tissue remodeling, angiogenesis and transformation in the development of cells, organs, and organisms.

Most ETS proteins, except GABPα, can recognize and bind to their unique EBS on the promoters/enhancers of downstream targets as a monomer (Karim *et al*, 1990). However, the transcriptional activity of ETS proteins is modulated by other factors/partners (Bhat and Papas, 1994; Crepieux *et al*, 1994). Many ETS transcription factors have been shown to interact with *cis*-regulatory elements present in target genes and function to activate or repress transcriptional activity (Li *et al*, 2000b; Mavrothalassitis and Ghysdael, 2000). For example, Elk-1 can recognize and bind to the E74 promoter element as a monomer (Rao *et al*, 1992b), but requires serum response factor (SRF) to bind an adjacent motif in order to form a stable complex (Rao *et al*, 1992a and 1993a). ETS-1 can recognize and bind PEA3 element of the Polyomavirus enhancer but efficient transactivation is dependent upon AP-1 binding the adjacent PEA1 site (Wasylyk *et al*, 1990). Both ETS-1 and ETS-2 recruit CBP/p300, an adaptor protein in the basal transcriptional machinery such as TFIID, to activate the MMP promoter (Jayaraman *et al*, 1999; Watabe *et al*, 1998). Besides, ETS-1 and CBP/p300 can form a stable complex which has the properties of which possesses histone acetyltransferases (HAT) activity in a DNA independent manner (Yang *et al*, 1998). In contrast to the synergistic activation, some ETS proteins exert repression instead of activation of specific target genes when bind to other transcription factors. For example,

MafB, an AP-1 like protein, interacts with ETS-1 and inhibits ETS-1 mediated transactivation of the transferrin receptor gene in a DNA-dependent manner that is necessary for erythroid differentiation (Sieweke *et al*, 1996). PU.1 also functions as a repressor when binding to GATA-1, a zinc finger transcription factor required for erythroid differentiation (Rekhtman *et al*, 1999). Moreover, when the Daxx protein or EAP1 (Ets 1 Associated Protein 1) binds to ETS-1, the interaction causes transcriptional repression on *MMP1* and *Bcl2* activation *in vitro* (Li *et al*, 2000a). Thus the regulation of genes by ETS proteins is complex and probably involves a variety of other factors depend on the specific genes, the ETS factors and the cellular context.

Activity of ETS family proteins is also regulated by phosphorylation. *ETS-1* is most abundant in lymphoid cells (Chen *et al*, 1985; Pognonec *et al*, 1988; Seth and Papas, 1990) while *ETS-2* is more ubiquitously expressed (Bhat *et al*, 1987). Stimulation of T and B lymphocytes by mitogens results in a rapid phosphorylation of the ETS-1 (Pognonec *et al*, 1990) and ETS-2 proteins (Fujiwara *et al*, 1988 and 1990). Phosphorylation of the EDB domain inhibits the binding activity of ETS-1 and ETS-2, however phosphorylation of the pointed domain increases transactivation potential and the half-life of ETS-2, suggesting that ETS-1 and ETS-2 may be inactivated by mitogenic signals (Fujiwara *et al*, 1988; Pognonec *et al*, 1990).

In addition, the expression patterns of *ETS* genes are consistent with the roles in cellular proliferation, differentiation and/or embryonic development. *ETS-2* is widely expressed (Bhat *et al*, 1987) and the reduction of *ETS-2* by injecting antisense *ETS-2* oligonucleotides can prevent the breakdown of the germinal vesicle, implicating ETS-2 in cell regulation (Bhat *et al*, 1987; Chen *et al*, 1990). During embryogenesis, *ETS-2* expression is high in newly forming cartilage (Maroulakou *et al*, 1994) and overexpression of *ETS-2* results in skeletal abnormalities.(Sumarsono *et al*, 1996).

Therefore, the regulation of *ETS* target genes depends upon a number of factors. Firstly, the specific interaction between specific DNA consensus binding site and the ETS family member. Secondly, the interaction between ETS and other transcription factors or accessory proteins in the context of the target promoter. Thirdly, the phosphorylation of ETS proteins due to activation of kinase pathways which affects gene transactivation and protein stability. Finally, the temporal and spatial patterns of expression of the *ETS*

genes in cellular processes. All these factors affect the activities of ETS transcription factors on the regulation of downstream target genes which are critical to cellular and multi-cellular functionality.

1.4-3 Expression and Function of ETS Family in Development

1.4-3.1 Normal Development

The large family of ETS transcription factors is involved in a variety of mammalian developmental processes at the cellular, tissue and organ levels through their roles in cellular proliferation, differentiation, migration, apoptosis and cell-cell interactions. During the earliest stages of embryonic development, the ETS factors impact greatly in haematopoiesis and vasculogenesis/angiogenesis. At the later stage of embryonic or in adult development, they are usually involved in tissue and organ development and are expressed in a variety of tissues including haematopoietic tissue, vasculature, brain and central nervous system, mammary gland, endometrium, ovaries, testes, kidneys and lungs (Maroulakou and Bowe, 2000). The gene expression patterns of some selected ETS factors in mouse development were summarized in Table 1.6.

To further the understanding of ETS transcription factors in normal development, gene targeting and knockout mice technologies provide good tools to study their physiological functions. For example, the *TEL-1* gene knockout in mouse has been shown to cause embryonic lethality (Wang *et al*, 1997). This has revealed that *TEL-1* is essential for the formation and/or maintenance of vascular networks and instead of angiogenesis in yolk sac (Wang *et al*, 1997). *ER81* deficient mice exhibit postnatal lethality by 5 weeks of age, probably due to neural defects because the mutant mice only have interneurons and do not have direct functional connections with motor neurons (Arber *et al*, 2000). This suggested that ER81 is essential for neural connectivity related to motor neurons (Arber *et al*, 2000). *ETS-2* targeted mutant mice present with early embryonic lethality with a defect in extraembryonic trophectoderm which is one of the first tissues in which *ETS-2* is highly expressed (Yamamoto *et al*, 1998). This showed that the earliest terminally differentiated lineage in extraembryonic

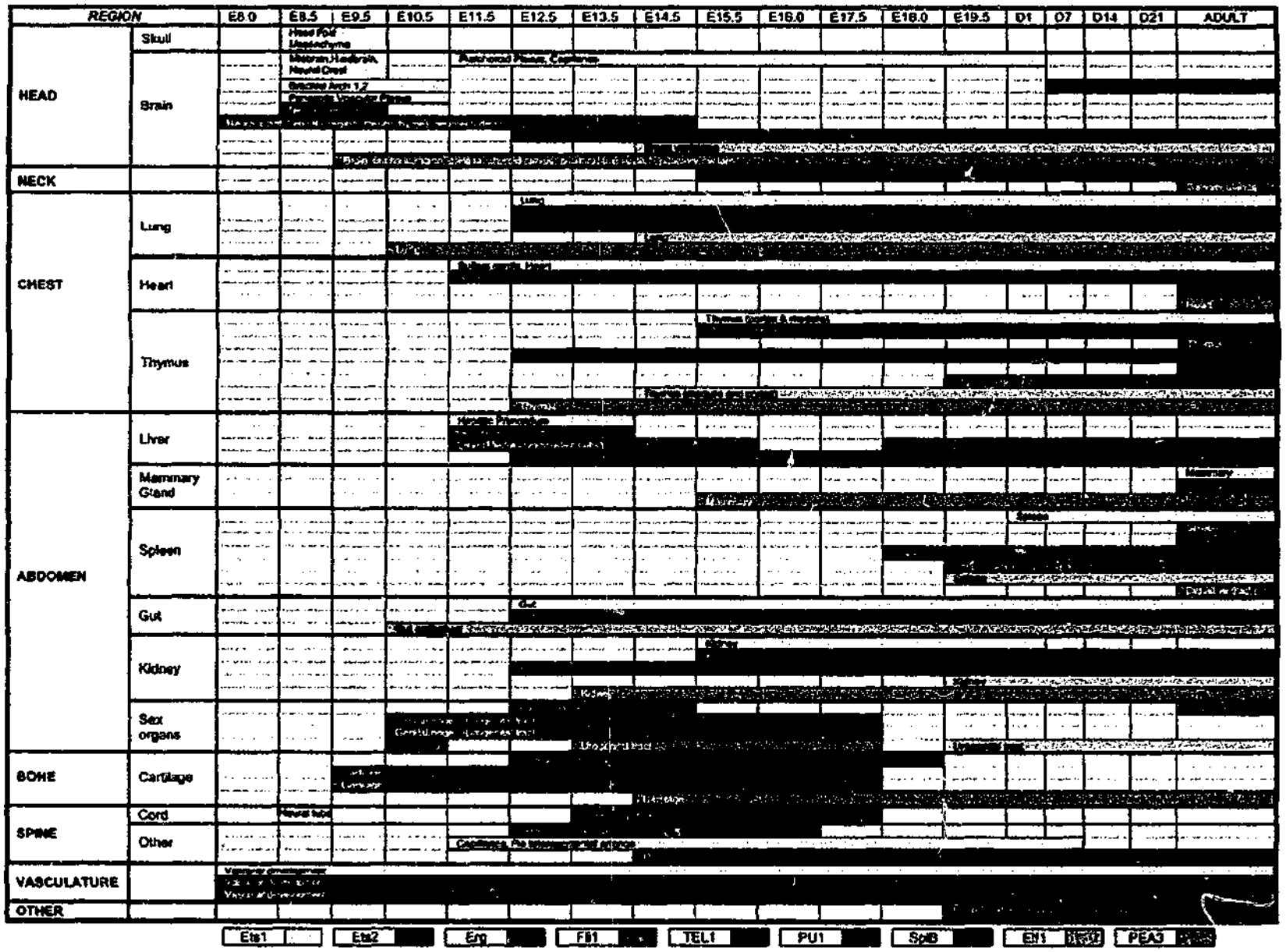


Table 1.6 Regions of ETS transcription factor expression during murine development. E = embryonic day; D = postnatal day. Expression ETS transcription factors are color-coded as follows: *ETS-1* (yellow); *ETS-2* (dark blue); *ERG* (green); *Fli*-1 (light blue); *TEL-1* (black); *PU.1* (maroon); *SpiB* (dark yellow); *Elf-1* (gray); and *PEA3* (pink) (From Maroulakou *et al*, 2000. *Oncogene*. **19**:6432-42.)

trophectoderm occurred in *ETS-2* deficient mice (Yamamoto *et al*, 1998). Apart from the above reports, most of the *ETS* gene knockouts generated to date have shown that the ETS transcription factors are important in haematopoiesis (Bartel *et al*, 2000)

1.4-3.2 Hematopoietic Development

The ETS family of transcription factors have been suggested to function as key regulators of haematopoeisis. Among these, PU.1 is a major regulator of myelo-lymphopoiesis. The disruption of the *PU.1* gene results in haematopoietic defects in mice. The *PU.1* knockout-mouse models demonstrated an absence of normal B cells and macrophages, defects in granulopoiesis and myelopoiesis, and aberrant T-lymphocyte development (McKercher *et al*, 1996; Scott *et al*, 1994; Tondravi *et al*, 1997). Spi-B is similar to PU.1 in terms of structural homology and ability to transactivate PU.1 target genes *in vitro* (Ray *et al*, 1992; Ray-Gallet *et al*, 1995). However, compared with *PU.1* knockout mice, *Spi-B* deficient mice exhibit a mild phenotype in haematopoietic defects, suggesting there may be functional compensation by *PU.1* and/or other ETS family members (Garrett-Sinha *et al*, 1999). *ETS-1* was shown to be an intermediate regulator of lymphoid development. The *ETS-1* knockout mice showed that ETS-1 is essential in the differentiation of all lymphoid lineages (Bories *et al*, 1995; Muthusamy *et al*, 1995). Furthermore, the absence of NK and NK T cells in *ETS-1* deficient mice indicated that ETS-1 is important for the development of functional NK and T cells (Walunas *et al*, 2000). Other ETS family members such as FLI-1 and TEL-1 display distinct and/or overlapping functions in vasculo/angiogenesis, haemostasis and haematopoiesis. The analysis of recently developed targeted mutants has revealed an essential role for *FLI-1* in megakaryopoiesis, haemotasis, and vascular integrity (Hart *et al*, 2000; Spyropoulos *et al*, 2000). Besides, the generation of mice carrying targeted disruption of the *Tel-1* gene demonstrated essential role for Tel-1 not only in early embryonic angiogenesis but also required for normal bone marrow haematopoiesis, erythropoiesis, and myelopoiesis (Wang *et al*, 1998). Different from the role of PU.1 in haematopoiesis, Tel-1 requires a suitable bone marrow microenvironment for haematopoiesis (Bartel *et al*, 2000).

1.4-3.3 Viral Infectious Cycle

ETS family members have also been shown to regulate viral genes. ETS-1 and ETS-2 activate the HTLV-1 LTR in T-cells and leads to the synthesis of TAX1. This viral protein is an initiator of viral replication which can further synergise with ETS-1 to regulate HTLV-1 replication (Bosselut *et al*, 1990; Gitlin *et al*, 1990). *ELF-1* appears to mediate HIV-2 induction following activation of T-cells (Leiden *et al*, 1992) while the interaction between ETS-1 and USF-1 is required for maximal transcriptional activity of the HIV-1 LTR in T cells (Sieweke *et al*, 1998). Induction of the HSV ICP4 promoter by the viral particle protein VP16 induces *GABPα* (Thompson *et al*, 1992). In addition, Moloney murine sarcoma virus is regulated by ETS-1 (Gunther *et al*, 1990), PEA3 activates early gene expression in polyoma virus infection (Wasylyk *et al*, 1989) and the simian virus 40 enhancer by *PU.1* (Klemsz *et al*, 1990). These examples emphasize the importance of *ETS* genes in the cell, since viruses take advantage of cellular machinery to replicate and efficiently induce gene expression.

1.4-4 The ETS Family in Transformation and Oncogenesis

1.4-4.1 Retroviral Activation of *ETS* Genes

The aetiology of many cancers involves abnormal expression of *ETS* genes which are induced or activated by virus. E26 is an acute avian leukaemia virus which causes mixed erythroid and myeloid leukaemia in avian species (Metz *et al*, 1991a) This virus has transduced the two transcriptional activator-type oncogenes *v-myb* and *v-ets* as a nuclear fusion protein, vMyb-vEts (Metz *et al*, 1991a). The DNA binding domains of v-Myb and v-ets as well as the v-myb transactivation domain need to be present in a single molecule to induce leukaemia (Metz *et al*, 1991b). Other cancers have been shown to be due to *ETS* gene activation by proviral insertion. To date, two members of the *ETS* gene family have shown to be insertionally activated by proviruses, resulting in erythroleukaemia. The *Fli-1* locus is rearranged in 75% of erythroleukaemias induced by the Friend murine leukaemia virus (F-MuLV) (Ben-David *et al*, 1991). In addition, *Spi-1* (*PU.1*) locus is rearranged in 95% of erythroid tumours caused by FV-P and FV-

A, which are complexes of spleen focus-forming virus (SFFV-P and –A) and F-MuLV (Moreau-Gachelin *et al*, 1988, 1989).

1.4-4.2 Chromosomal Translocations of *ETS* Genes in Cancers

Chromosomal translocations are frequently associated with hematological malignancies and human sarcomas. Chimaeric proteins that contain domains of ETS proteins have been identified in Ewing tumour and certain types of leukaemias such as B-type childhood acute lymphoblastic leukaemia (ALL).

In 85% of cases of Ewing's sarcoma, there is a t(11;22)(q24;q12) translocation when the EDB domain of *FLI-1* is fused to the *EWS* gene. Further 10% of these tumours there is a t(21;22)(q22;q12) translocation which involves the EDB domain of *ERG* (Delattre *et al*, 1992; Sorensen *et al*, 1994). Other rare Ewing tumour cases are due to translocations which fuse *EWS* to other *ETS* domains of *ETV-1*, *E1AF* or *FEV* (Jeon *et al*, 1995; Kaneko *et al*, 1996; Peter *et al*, 1997). Certain types of human leukaemia express fusion proteins that contain domains of the ETS protein ETV6/TEL or ERG. For example, the fusion protein found in chronic myeloid leukaemia (CML) associated with a t(6;21) chromosomal translocation, is structurally analogous to the EWS-ETS proteins in Ewing tumours. It consists of the EDB domain of ERG and the N-terminal domain of a EWS-related protein TLS/FUS (Ichikawa *et al*, 1994; Panagopoulos *et al*, 1994). This translocation is predicted to cause a loss of specificity of the EDB domain and suggests that different modulation of ETS DNA-binding activity by different transactivation domains for EWS or TLS/FUS could result in different cancers (Ichikawa *et al*, 1994; Panagopoulos *et al*, 1994).

A subset of chronic myelomonocytic leukaemias is associated with a translocation t(5;12) which generates a fusion product composed of *TEL* and the *PDGFβ* receptor (Golub *et al*, 1994). In ALL, the *TEL* and *AML1* genes are fused in a t(12;21) translocation (Golub *et al*, 1995). In these two cases, the PNT domain of the *TEL* gene is retained and proposed to function in self-association, leading to altered activity of either *AML1* or the *PDGFβ* receptor. *TEL* has also been shown to contribute to other

myeloid and lymphoid leukaemias where the chromosomal translocation t(12;22)(p13;q11) fuses *TEL* gene to the putative transcription factor *MN1* which forms *MN1-TEL* and *TEL-MN1* (Buijs *et al*, 1995). It has not been determined which fusion gene is oncogenic.

Chromosome translocations can alter many properties of a gene, including its expression pattern and protein structure. In fusion proteins, the isolated domains of the fused proteins appear to acquire new biological roles while retaining their molecular function. Thus the phenotypes observed may not directly relate to the normal biological functions of the wild-type *ETS* gene. These phenotypes should also be considered by the ETS translocated partners (e.g. EWS or TLS/FUS) which may exert different regulatory pathways in gene regulations.

1.4-4.3 Overexpression of ETS Factors in Tumours

Transformation may also result from *ETS* overexpression. *ETS-1* is amplified in myelodysplastic syndrome (Ohyashiki *et al*, 1990) and extra gene dosage of *ETS-2* in Down's syndrome may increase susceptibility to leukaemia (Sacchi *et al*, 1988). The *ETS-2* mRNA level increases in an acute nonlymphoblastic leukaemia (Santoro *et al*, 1992). ETS proteins have also been found to be overexpressed in mammary epithelial carcinoma cells. These ETS proteins include ETS members of the PEA3 family, PEA3, ER81, and ERM (Monte *et al*, 1995; Chen *et al*, 1996), as well as ELF3/ESX/ESE-1 (Tymms *et al*, 1997; Chang *et al*, 1997; Oettgen *et al*, 1997). The expression of *ER81* seems to be restricted to the estrogen/progesteron negative type of breast cancers, whereas increased levels of *ELF3* are only found in tumours that are positive for the transmembrane receptor kinase, *HER2/neu* (Chang *et al*, 1997; Oettgen *et al*, 1997). The *HER2/neu* promoter is transactivated by *ELF3* which suggests that *HER2/neu* over-expression in 20-30% of primary breast cancers may be secondary to *ELF3* overexpression.

1.4-5 Biology of *FLI-1*

1.4-5.1 FLI-1 is a Member of the ETS Family of Transcriptional Factors

The proto-oncogene, mouse *Fli-1*, was first identified as a common site for retroviral integration in Friend virus-induced erythroleukaemias (Ben-David *et al*, 1990, 1991). Of primary interest in this study is the ETS transcription factor, FLI-1, which is the most common ETS member fused to the RNA binding domain of EWS in Ewing's sarcoma and primitive neuroectodermal tumours (Delattre *et al*, 1992).

Subsequent to the cloning of mouse *Fli-1*, the human *FLI-1* gene was isolated by sequence homology (Prasad *et al*, 1992; Watson *et al*, 1992). Localization analysis revealed the *FLI-1* gene is within 240 kb of the *ETS-1* locus on mouse chromosome 9 and on human chromosome 11q23 (Ben-David *et al*, 1991; Watson *et al*, 1992). Human and mouse *Fli-1* contains nine exons which extend over approximately 120 kb (Selleri *et al*, 1994) and encode two proteins, p51 (452 a.a.) and p48 (419 a.a.). A comparison of the amino acid sequences of human FLI-1 revealed 97% homology to mouse Fli-1 (Prasad *et al*, 1992). The human gene encodes a 3.5 kb transcript with two alternatively spliced forms, *FLI-1* and *FLI-1b*, which with and without first exon respectively and are differentially expressed between cell types (Prasad *et al*, 1992, 1998). These spliced forms are regulated by two independent promoters and promoter assays revealed that the *FLI-1b* promoter shows stronger transcriptional activation compared to the *FLI-1* promoter in various hematopoietic cell lines (Prasad *et al*, 1998). This suggests that the regulation of *FLI-1* is complex at the transcriptional levels.

ERG, another ETS member, contains high identity to *FLI-1* and is involved in ES/PNET translocations. Both FLI-1 and ERG contain two regions designated as the 5' and 3' ETS domains or helix 1-loop-helix 2 (H-L-H) structures which are homologous to those found in the ETS-1 and ETS-2 proteins (Fig.1.8) (Rao *et al*, 1993c). However, a FLI-1 specific region (FLS), which is absent in the ERG protein, has been localized within the region encompassing amino acids 205-292 (Fig. 1.8). The ATA domain which consists of FLS and 5' ETS domains is responsible for transcriptional activation (Rao *et al*, 1993c). The CTA domain (amino acids 402-452) at the C-terminal region of FLI-1 is

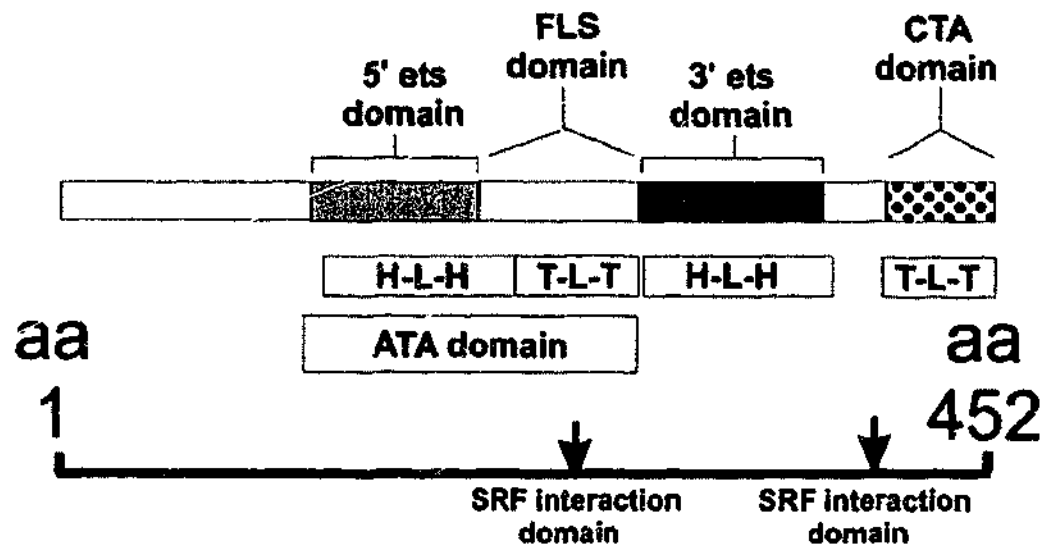


Fig.1.8 Diagram of the functional domains located within the FLI-1 protein. Both human and murine FLI-1/Fli-1 consist of 452 amino acids (aa) which contain the following domains: ATA = amino-terminal transcriptional activation domain, FLS = FLI-1 specific domain, CTA = carboxy-terminal transcriptional activation domain, H-L-H = helix-loop-helix structure, and T-L-T = turn-loop-turn structure. The positions of the SRF interactive domains are shown by arrows. (From Truong and Ben-David, 2000. *Oncogene.* **19**:6482-89).

also involved in transcriptional activation. However, in terms of transcriptional control, the ATA domain alone, but not the CTA domain alone, results in a significant increase in the transcription of downstream target genes compared with the wild-type FLI-1 protein (Rao *et al*, 1993c). This suggests that the CTA region may serve as a transcriptional activator and repressor in FLI-1 transcriptional activity (Rao *et al*, 1993c).

Furthermore, FLI-1 and ERG show high identity in EDB domain (Fig.1.7) (Ben-David *et al*, 1991; Prasad *et al*, 1992). The C-terminal domain of FLI-1 protein (amino acids 277-360) shows 98% homology with ERG whereas with other ETS proteins the homology ranges from 35 to 72% (Watson *et al*, 1988). Because of the 98% identity in their EDB domains, the human FLI-1 and ERG proteins are suggested to bind to the same DNA binding sequence which is ACCGGAAG/aT/c (Reddy *et al*, 1991; Mao *et al*, 1994).

The N-terminal region of FLI-1 protein (amino acids 121-196) showed 82% identity with ERG protein whereas it shows 59-60% similarity with ETS-1 and ETS-2 proteins (*Prasad et al*, 1992). This region (the transactivation domain) is conserved in only five genes; *FLI-1*, *ETS-1*, ETS-2, *GABPα* and *ERG*, suggesting a common biological function which is shared among these genes (Watson *et al*, 1988; Prasad *et al*, 1992) but the actual functions exerted from each ETS proteins also depends on the co-factors interaction (see Section 1.4-2). Hence, this 5' domain may serve as a ETS transcriptional activator domain or protein-protein interaction site in all these ETS proteins.

1.4-5.2 Normal Biological Functions of *FLI-1*

Insight into the biological function of FLI-1 has been addressed by examining its expression pattern during development, its DNA binding specificity and the phenotypes of mutant mice. The *Fli-1* gene is highly expressed in endothelial cells and in all haematopoietic tissues, especially lymphoid tissues (Ben-David *et al*, 1991; Klemsz *et al*, 1993). During embryogenesis, murine *Fli-1* is expressed at E8.5 in the blood islands

of the extra embryonic visceral yolk sac in a pattern consistent with its expression in putative erythroid/endothelial precursors or hemangioblasts present at this time (Melet *et al*, 1996). Later in gestation (11.5-15.5 days postcoitus, dpc), *Fli-1* is expressed in the developing vasculature and within the liver, coincident with fetal haematopoiesis (Melet *et al*, 1996). *Fli-1* deficient mice demonstrated a loss of vessel integrity after embryonic day 11 and a partial block in megakaryocyte differentiation (Hart *et al*, 2000). In addition, *Fli-1* deficient mouse embryos were found to hemorrhage from the dorsal aorta to the lumen of the neural tube and ventricles of the brain (hematorrhachis) on embryonic day 11.0 (E11.0) and die by E12.5 (Spyropoulos *et al*, 2000). This suggests a role for *Fli-1* in the regulation of haematopoiesis and haemostasis (Spyropoulos *et al*, 2000).

Many genes expressed in the hematopoietic compartment contain a consensus FLI-1 binding site in their transcriptional regulatory elements (Leiden *et al*, 1994; Hagman *et al*, 1994; McCracken *et al*, 1994). For example, the retinoblastoma (*Rb*) gene, which is involved in the development of mature erythrocytes, contains a FLI-1 consensus binding site within its promoter (Zhang *et al*, 1993; Tamir *et al*, 1999). The FLI-1 protein binds to this site within the *Rb* promoter and transcriptionally represses *Rb* expression (Tamir *et al*, 1999). This negative regulation by FLI-1 on *Rb* expression could be important in erythroid progenitor cell differentiation (Tamir *et al*, 1999). Besides, FLI-1 has been shown to transactivate a number of genes involved in vasculogenesis, hematopoiesis, and cell adhesion, including the *endothelial-specific vascular endothelial-cadherin* (*VE-CAD*) (Gory *et al*, 1998), *Tek/Tie-2* (Dube *et al*, 1999), *intercellular cellular adhesion* (*I-CAM*) genes (de Launoit *et al*, 1998), and the *megakaryocyte-specific genes glycoprotein IIB* (*GpIIb*) (Zhang *et al*, 1993), *GplX* (Bastian *et al*, 1996), *Von Willebrand factor* (*VWF*) (Schawachtgen *et al*, 1997), and *platelet factor 4* (*PF4*) (Lemarchandel *et al*, 1993).

Homologs of the mammalian *FLI-1* gene have been identified in several other vertebrates, including quail (Mager *et al*, 1998), Xenopus (Meyer *et al*, 1995), and zebrafish (Brown *et al*, 2000). Each of these *Fli-1* homologs are expressed during the development of endothelial and hematopoietic cells. In zebrafish, *Fli-1* is expressed in the mesoderm and in areas of early haematopoiesis and vasculogenesis (Brown *et al*, 2000). In chicken, the *Fli-1* gene is expressed in the neural crest cells and mesoderm-

derived cells, endothelial cells as well as intermediate and splanchnopleural mesoderm (Mager *et al*, 1998). *Erg*, another closely related gene to FLI-1, was also identified in migrating neural crest cells and in mesodermal tissues (Vlaeminck-Guillem *et al*, 2000). This suggests that ERG and FLI-1 may have related functions in these tissues. Interestingly, birds have two spliced variants of *Fli-1* as also observed in mouse and human, mostly likely arising from two different promoters (Mager *et al*, 1998). In Xenopus, the *XI-Fli-1* gene is also expressed in a restricted pattern of neural crest cells during embryogenesis (Meyer *et al*, 1993). Overexpression of the *Fli-1* gene in Xenopus results in severe developmental anomalies, which affect anteroposterior and dorsoventral polarities, optic cup formation, head cartilage morphogenesis, and erythrocyte differentiation (Remy *et al*, 1996). This was correlated with modifications of cell adhesion properties and an increased number of apoptotic cells during early development (Remy *et al*, 1996; Goltzene *et al*, 2000).

1.4-5.3 *FLI-1* Gene in Tumours and Human Diseases

Overexpression of the *FLI-1* gene is associated with mouse erythroleukaemias. Translocations including the *FLI-1* gene result in human Ewing's sarcoma (see section 1.2) (Ben-David *et al*, 1991; Delattre *et al*, 1992). Indeed, *Fli-1* was originally cloned from the integration site of the Friend murine leukaemia virus (F-MuLV) in a murine erythroleukaemia cell line (Ben-David *et al*, 1991). Moreover, *FLI-1* is expressed at high levels in many human erythroleukaemia cell lines (Klemsz *et al*, 1993). The overexpression of *FLI-1* gene in primary erythroblasts has been shown to deregulate the normal balance between differentiation and proliferation rate in these cells (Pereira *et al*, 1999). Thus, the aberrant *FLI-1* activation increase malignant transformation occurred at the onset of F-MuLV-induced erythroleukaemia may due to promoting of uncontrolled cell division. Furthermore, overexpression of *FLI-1* in fibroblasts has been shown to inhibit apoptosis (Yi *et al*, 1997). The high expression of FLI-1 or its fusion proteins observed in ES/PNET cells has been shown to inhibit cell apoptosis in either serum deprivation or by treatment with calcium ionophore media (Yi *et al*, 1997). Further, molecular studies found that FLI-1 binds to the ETS consensus site within the *Rb* promoter and transcriptionally represses *Rb* expression (Tamir *et al*, 1999) but up-regulates *Bcl-2* in erythroblasts (Pereira *et al*, 1999). All these data indicate that

activation of *FLI-1* gene in erythroid cells can promote cell cycle progression and cell survival. This could be one potential mechanism for erythroid progenitor cell differentiation that is specifically deregulated during F-MuLV-induced erythroleukaemia.

In addition, the majority of human chromosomal translocations that result in Ewing's sarcoma and related tumours involve the fusion of the *EWS* gene to the *FLI-1* gene. This results in chimaeric proteins comprising the N-terminal region of *EWS* and the C-terminal region (ETS-domain) of *FLI-1* (Zucman *et al*, 1993). This translocation changes the FLI-1 binding characteristics and the expression pattern since the EWS transactivation domain and promoter in this fusion protein change the normal functions of FLI-1.

1.5 Functions of EWS/FLI-1 Fusion Protein in ES/PNET

1.5-1 EWS/FLI-1 is Potent Transforming Oncogene

Approximately 85% of ES/PNET have t(11;22) translocation and produce EWS/FLI-1 fusion protein (Zucman *et al*, 1993, Sorensen *et al*, 1994), suggesting that this protein has a key role in the pathogenesis of these tumours (see Section 1.2-2). The EWS/FLI-1 fusion protein has also been shown to efficiently transform NIH3T3 fibroblast cells, forming colonies in soft agar and tumours in nude mice (May *et al*, 1993a, b). In contrast, normal FLI-1 does not transform NIH3T3 fibroblast cells (May *et al*, 1993b). These data suggest that EWS/FLI-1 fusion protein is functionally distinct from FLI-1 and provides further evidence for its role in ESFT aetiology. On the other hand, deletion of either *EWS* or the EDB domain of *FLI-1* completely abrogates oncogenic potential (May *et al*, 1993a). Therefore, the requirement for both components of the EWS/FLI-1 chimaera suggests that EWS/FLI-1 has a dominant mode of action and does not act primarily by interfering with either normal cellular *EWS* or *FLI-1* gene. Indeed, in ES/PNET, the weak transactivation domain of FLI-1 is substituted by the potent transactivation domain of EWS and thereby resulting a stronger transactivator, EWS/FLI-1, dysregulates the expression of subset of primary targets that ultimately

induce uncontrolled cell growth. Although the expression of EWS or EWS/FLI-1 is ubiquitously expressed under the control of endogenous EWS promoter, the EWS/FLI-1 fusion protein does not transform all cells observed in ES/PNET phenotypes. Studies found that neither Rat1 cells nor particular sub-strains of NIH3T3 cells are transformed by EWS/FLI-1 (May *et al*, 1993a). These indicate that the EWS/FLI-1 fusion protein needs to interact with other cellular factors to transform and/or transformation resistant cells may express proteins that inhibit the effect of EWS/FLI-1.

1.5-2 EWS/FLI-1 Has Biochemical Characteristics of a Potent Transcription Factor

The EWS/FLI-1 chimaeric protein contains the DNA-binding domain of FLI-1 and the amino terminal region of EWS. This fusion protein acts as a potent transcription factor since the amino terminal domain of *EWS* gene is a strong transcriptional activation domain (May *et al*, 1993b, Lessnick et al, 1995). The latter has been shown by coupling this transactivational domain of EWS to a yeast *GAL4* DNA-binding domain, in which case the amino terminal domain of EWS was able to activate reporter genes more efficiently than the transcriptional activation domain of *FLI-1* gene (May *et al*, 1993b; Bailly *et al*, 1994). EWS/FLI-1 or EWS/ERG fusion proteins which share high homology in their EDB domains have also been shown to efficiently activate reporter constructs (Ohno *et al*, 1993, 1994). Moreover, generation of chimaeric FLI-1 fusion proteins with known heterologous transcriptional activation domains yielded chimaeric FLI-1 fusions, that in some instances, could also transform NIH3T3 cells (Lessnick *et al*, 1995). This indicates that the transformation induced by EWS/FLI-1 maybe due to higher transactivational transcription activity on activation of FLI-1 downstream targets. Moreover, there is evidence showing that EWS/FLI-1 displays biochemical characteristics that are common to transcription factors. For example, similar to FLI-1 which has NLS at the EDB domain, the EWS/FLI-1 proteins also localizes to the nucleus in ES/PNET cells (May *et al*, 1993b, Bailly *et al*, 1994). It can bind DNA in a site-specific manner by FLI-1 ETS motif (Mao *et al*, 1994). Finally, it requires both the transactivation and the EDB domains to modulate downstream targets. Mutation in either of these domains will stop the transformation activity (Lessnick *et al*, 1995).

EWS/FLI-1 and FLI-1 are transcription factors and share same EDB domains. Why does EWS/FLI-1 but not FLI-1 transforms NIH3T3 cells? One hypothesis is that EWS/FLI-1 has a stronger transcriptional activation activity which allows EWS/FLI-1 protein to modulate target genes more extensively than FLI-1 (May *et al*, 1997a, 1997b; Hahm *et al*, 1999). One study demonstrated that EWS/FLI-1 could activate a reporter construct with an order of magnitude greater than FLI-1. ETS proteins have also been shown to form heteromeric complexes with other factors during the activation of target genes which may be unable to form with the EWS N-terminal domain. These intermolecular protein-protein interactions are critical to the specificity of certain ETS proteins (Waslyk *et al*, 1993). For example, the ETS protein SAP-1 requires an additional factor (SRF) to productively activate the *c-FOS* gene (Dalton *et al*, 1992). FLI-1, unlike EWS/FLI-1, requires SRF to bind the serum responsive element (SRE) of c-FOS (Magnaghi-Jaulin *et al*, 1996). Both FLI-1 and EWS/FLI-1 proteins can form complexes on SREs on *EGR1* promoter in the presence of SRF (Watson *et al*, 1997), but EWS/FLI-1 can bind to more SREs and form more stable complexes (Watson *et al*, 1997). These data indicate that there are intrinsic differences in the binding of EWS/FLI-1 and FLI-1 proteins to distinct ETS sequences. Hence, the EWS/FLI-1 protein may not be as constrained in its target selection and could potentially up-regulate genes without cofactors that are essential for activation by FLI-1.

1.5-3 EWS/FLI-1 Downstream Targets

Although EWS/FLI-1 is clearly a transcriptional activator, the mechanism by which EWS/FLI-1 promotes cellular growth is largely unknown. To determine genes potentially important in the development of ES/PNET, EWS/FLI-1 transformed and *FLI-1* transfected NIH3T3 cells were subjected to RNA differential analysis. This resulted in the identification of several interesting genes (Table1.7) (Braun *et al*, 1995) which have been implicated in the control of cell growth, differentiation, and oncogenesis. For example, EWS/FLI-1 upregulates *Stromelysin-1*, a member of the metalloproteinase family that digests a variety of extracellular matrix proteins and contributes to the ability of tumour cells to invade the surrounding connective tissue (Braun *et al*, 1995). Since tandem ETS binding motifs have been shown to be

EWS/FLI-1 Downstream Targets	References
Activation	
EAT1	Braun *et al*, 1994
EAT2	Braun *et al*, 1994
	Thompson *et al*, 1996
EAT3	Braun *et al*, 1994
EAT4	Braun *et al*, 1994
P-450	Braun *et al*, 1994
CK 15	Braun *et al*, 1994
Stromelysin-1	Braun *et al*, 1994
c-myc	Bailly *et al*, 1994
c-fos	Magnaghi-Jaulin *et al*, 1996
Egr-1	Watson *et al*, 1997
mE2-C	Arvand *et al*, 1994
Down-regulation	
ERT-1	Braun *et al*, 1994
ERT-2	Braun *et al*, 1994
TGFβRII	Hahm *et al*, 1999

Table 1.7 Summary of known genes which have been shown to be activated or down-regulated by EWS/FLI-1.

responsive to ETS-1, ETS-2 and TEL in rat and human *stromelysin-1* promoters, the *stromelysin-1* gene may be a primary and direct target that responds to EWS/FLI-1 but not FLI-1. (Wasylyk *et al*, 1991, 1992; Fenrick *et al*, 2000). *Manic Fringe* (*MFNG*) has also regulated in these cells. *MFNG* is a member of the *Fringe* gene family encoding secreted signaling molecules instrumental in somatic development (Johnston *et al*, 1997; Yuan *et al*, 1997). Although ectopic MFNG expression does not completely recapitulate the effects of EWS/FLI-1 overexpression, it can render NIH3T3 cells tumorigenic in immunodeficient mice (May *et al*, 1997). Another gene, *EAT2* (*EWS/FLI-1 activated transcript 2*), has features of a signal transduction molecule, is activated in EWS/FLI-1 transformed cells (Thompson *et al*, 1996). An increase of *mE2-C*, a cyclin-selective ubiquitin-conjugating enzyme involved in cyclin B turnover and cycle control, was also observed (Arvand *et al*, 1998). *mE2-C* may be indirectly regulated by EWS/FLI-1 as it is also unregulated in v-ABL and c-myc transformed cells and *c-myc* is also increased in EWS/FLI-1 transformed NIH3T3 cells and in ES/PNET or related tumours (Bailly *et al*, 1994). The activation of *c-myc* is sufficient to induce cell cycle entry in quiescent cells (Eilers *et al*, 1991) and inhibition of *c-myc* expression leads to growth arrest and induction of differentiation (Biro *et al*, 1993; Heikkila *et al*, 1987; Prochownik *et al*, 1988). Other oncogenes such as *c-fos* and *Egr-1* may also be activated indirectly via a serum response element (Magnaghi-Jaulin *et al*, 1996; Watson *et al*, 1997). EWS/FLI-1 has been shown to bind the serum response element (SRE) and form a complex with the serum response factor (SRF) (Bailly *et al*, 1994; Magnaghi-Jaulin *et al*, 1996; Watson *et al*, 1997).

Some genes are also down-regulated in EWS/FLI-1 transformed cells (Table 1.7) (Braun *et al*, 1994). Suppression of *transforming growth factor–β type II receptor* (*TGFβR2*) gene, a putative tumour suppressor gene, appears to be direct since EWS/FLI-1 specifically binds to its promoter (Hahm *et al*, 1999). These data suggest that EWS/FLI-1 probably does not regulate all target genes involved in the aetiology of ES/PNET directly. It is more likely that EWS/FLI-1 interacts with the regulatory sequences of a small number of target genes which ultimately lead to transformation. Identification of these genes is a crucial step in determining the biochemical mechanism of EWS/FLI-1. The identification of downstream targets of EWS/FLI-1 can help

elucidate the biochemical mechanisms of EWS/FLI-1 transformation and open up avenues for future therapy of this tumour.

1.5-4 Altered Signaling Pathways in ES/PNET Cells

Although the mechanisms are unclear, alterations in a number of signaling pathways have been associated with ES/PNET. For example, EWS/FLI-1 induced transformation of fibroblasts requires IGF-IR and that some Ewing's sarcoma cell lines can produce insulin-like growth factor-I (IGF-I). This ligand then activates IGF-IR on the surface of other Ewing's cells (Scotlandi *et al*, 1996; Toretsky *et al*, 1997). The signaling pathway initiated by the binding of IGF-I to IGF-IR has subsequent downstream effects that inhibit apoptosis and may contribute to ES/PNET transformation. To further investigate the IGF-IR signaling pathway, a specific inhibitor to phosphoinositide 3-hydroxide kinase (*PI 3-K*) was used in ES/PNET cells to demonstrate that this effect was mediated by the PI 3-K and Akt pathway (Toretsky *et al*, 1999). A number of other pathways were altered in ES/PNET. These include the phospholipase C signaling pathway which was inhibited by *EWS/FLI-1* antisense oligonucleotides resulting in decreased growth of ES/PNET cells (Dohjima *et al*, 1999). In addition, the bFGF pathway was shown to maintain a malignant phenotype of Ewing's sarcoma cells through up-regulation of *EWS/FLI-1* expression (Girnita *et al*, 2000). Furthermore, the mitogen-activated protein kinase (MARK) signaling pathway was also activated in ES/PNET and other tumours with constitutional activation of either ERK1 or ERK2 (Sivaraman *et al*, 1997; Patton *et al*, 1998; Mandell *et al*, 1998; Sebolt-Lepold *et al*, 1999; Gioeli *et al*, 1999; Hosino *et al*, 1999; Silvany *et al*, 2000). Specific inhibitors or a dominant negative *Ras* reduced the ability of EWS/FLI-1 to transform fibroblasts *in vitro*.

These results suggest that many potentially oncogenic pathways are activated in ES/PNET and inhibition of these elements may improve disease prognosis. However, it will be important to address the events which result in activation of these pathways.

1.6 Inhibition of Cell Growth of ES/PNET

1.6-1 Potential Approaches for Reversing Tumour Phenotype in ES/PNET

EWS/FLI-1 acts as an aberrant transcription factor resulting in Ewing's sarcoma. Thus, to inhibit or reverse the transformed phenotype of ES/PNET cells, the action of this factor must be suppressed. This would be achieved by removal of the *EWS/FLI-1* fusion genes or proteins, by suppressing the action of EWS/FLI-1 on regulatory sequences of primary target genes, or by inhibiting the biological pathways which are activated by EWS/FLI-1 (see section 1.5-4).

1.6-2 Targeting at *EWS/FLI-1* mRNA

1.6-2.1 Antisense *EWS/FLI-1* mRNA

Expression of antisense *EWS* fusion transcripts resulted in a significant loss of endogenous EWS/FLI-1 and EWS/ERG proteins in Ewing's sarcoma cells (Ouchida *et al*, 1995; Kovar *et al*, 1996). These cells also showed loss of capability of anchorage independent growth and formation of tumours in nude mice than the parental Ewing's sarcoma cells (Ouchida *et al*, 1995; Kovar *et al*, 1996; Dohjima *et al*, 2000). Western blotting revealed that phospholipase Cbeta2 and beta3 (PLCbeta2, PLCbeta3), and also protein kinase C alpha and beta (PKCalpha, beta) were significantly reduced after transfection with antisense *EWS/FLI-1* transcript (Dohjima *et al*, 2000). These data suggest that the PLCbeta2 and PLCbeta3 are regulated by EWS/FLI-1 and may play a role in proliferation of Ewing's sarcoma cells (Dohjima *et al*, 2000). These reports also demonstrate that a certain threshold level of EWS-fusion products are required for the tumourigenicity of Ewing's sarcoma cells, emphasizing the potential for targeting EWS-fusion products as a therapy for the Ewing family of tumours.

1.6-2.2 Anti-sense Oligonucleotides of *EWS/FLI-1* Transcripts

A potentially more efficient approach to reduce the *EWS/FLI-1* mRNA is using antisense oligonucleotide of *EWS/FLI-1*. Oligonucleotides have the advantage of entering cells relatively easily and have the potential to be more specific (Crooket *et al* 1993; Stein *et al*, 1993). Antisense DNA oligomers are short sequences designed to be complementary to a unique site on the RNA transcript of a target gene. Antisense DNA is believed to inhibit translation through several mechanisms, including blocking ribosomal translation of the mRNA transcript (Haeuptle *et al*, 1986), triggering RNase H degradation of the mRNA (Walder *et al*, 1988), and interfering with RNA processing (Munroe, 1988). Thus, sequence-specific antisense oligodeoxynucleotides (ODN) have been used to block *EWS/FLI-1* expression in ES/PNET cells (Tanaka *et al*, 1997; Toretsky *et al*, 1997; Wang *et al*, 1999). These cells had significantly reduced growth and tumour formation rate *in vitro* and *in vivo* (Tanaka *et al*, 1997; Toretsky *et al*, 1997; Wang *et al*, 1999). These data further confirm that EWS/FLI-1 may play an important role in the tumourigenesis of ES/PNET.

1.6-3 Competition and Suppression of EWS/FLI-1 Transcriptional Activity

1.6-3.1 Suppression of EWS/FLI-1 Transcriptional activity by Specific Transcriptional Suppressor

An alternative mechanism to reverse the transformed phenotype induced by EWS/FLI-1 fusion protein is to introduce repressor proteins which can recognize the same DNA binding sites as EWS/FLI-1. For example, the t(2;13) chromosomal translocation in alveolar rhabdomyosarcoma tumours (ARMS) creates an oncogenic transcriptional activator by fusion of PAX3 DNA binding motifs to a COOH-terminal activation domain derived from the *FKHR* gene (Davis *et al*, 1994; Fredericks *et al*, 1995). Similar to EWS/FLI-1, the oncogenic potential of the PAX3-FKHR fusion protein is dependent on the FKHR transcriptional activation domain (Fredericks *et al*, 1995). Stable expression of an engineered PAX3-KRAB transcriptional repressor inhibits the

malignant phenotype of ARMS cells (Fredericks *et al*, 2000). The PAX3-KRAB repressor functions as a DNA-binding-dependent suppressor of the transformed phenotype of ARMS cells, probably via competition with the *PAX3-FKHR* oncogene and repression of target genes required for ARMS tumorigenesis (Fredericks *et al*, 2000).

1.6-3.2 Biology of KRAB Zinc Finger Proteins

The use of a DNA sequence specific repressor such as KRAB (Kruppel associated box) is a potential approach for therapy of ES/PNET. The KRAB domain is one of the most potent and widely distributed transcriptional repression domains identified in mammals (Margolin et al, 1994; Witzgall et al, 1994). Approximately one-third of the 300 to 700 Kruppel Cys_2His_2-type zinc-finger proteins in human contain a KRAB domain in their amino termini (Bellefroid *et al*, 1991). This regulatory domain consists of ~75 amino acid residues that have a repression effect on both basal and activated transcription when fused to a heterologous DNA binding domain (Margolin *et al*, 1994; Witzgall *et al*, 1994; Pengue *et al*, 1995; Vissing *et al*, 1995). The KRAB domain consists of KRAB-A and KRAB-B domains and is predicted to fold into two amphipathic helices (Fig.1.9) (Margolin *et al*, 1994). The KRAB-A module which consists of ~45 amino acid residues has the minimal repressional function in the KRAB domain. Substitutions for conserved residues at this KRAB-A module have been shown to abolish repression function of KRAB (Fig. 1.9) (Margolin *et al*, 1994). The repression effects can exert even when the DNA binding sites are situated at more than 3 kb upstream or downstream of the transcriptional initiation site (Deuschle *et al*, 1995; Moosmann *et al*, 1997). Furthermore, the silencing effect over short and long distances is not restricted to RNA polymerase II, since transcription by RNA polymerase III is also repressed (Moosmann *et al*, 1997). KRAB domains from more than ten factors have so far been demonstrated to be potent repressors, suggesting that this activity is a common property (Lim *et al*, 1998; Poncelet *et al*, 1998; Agata *et al*, 1999; Tekki-Kessaries *et al*, 1999; Dreyer *et al*, 1999; Han *et al*, 1999; Skapek *et al*, 2000; Peng *et al*, 2000a; Yano *et al*, 2000).

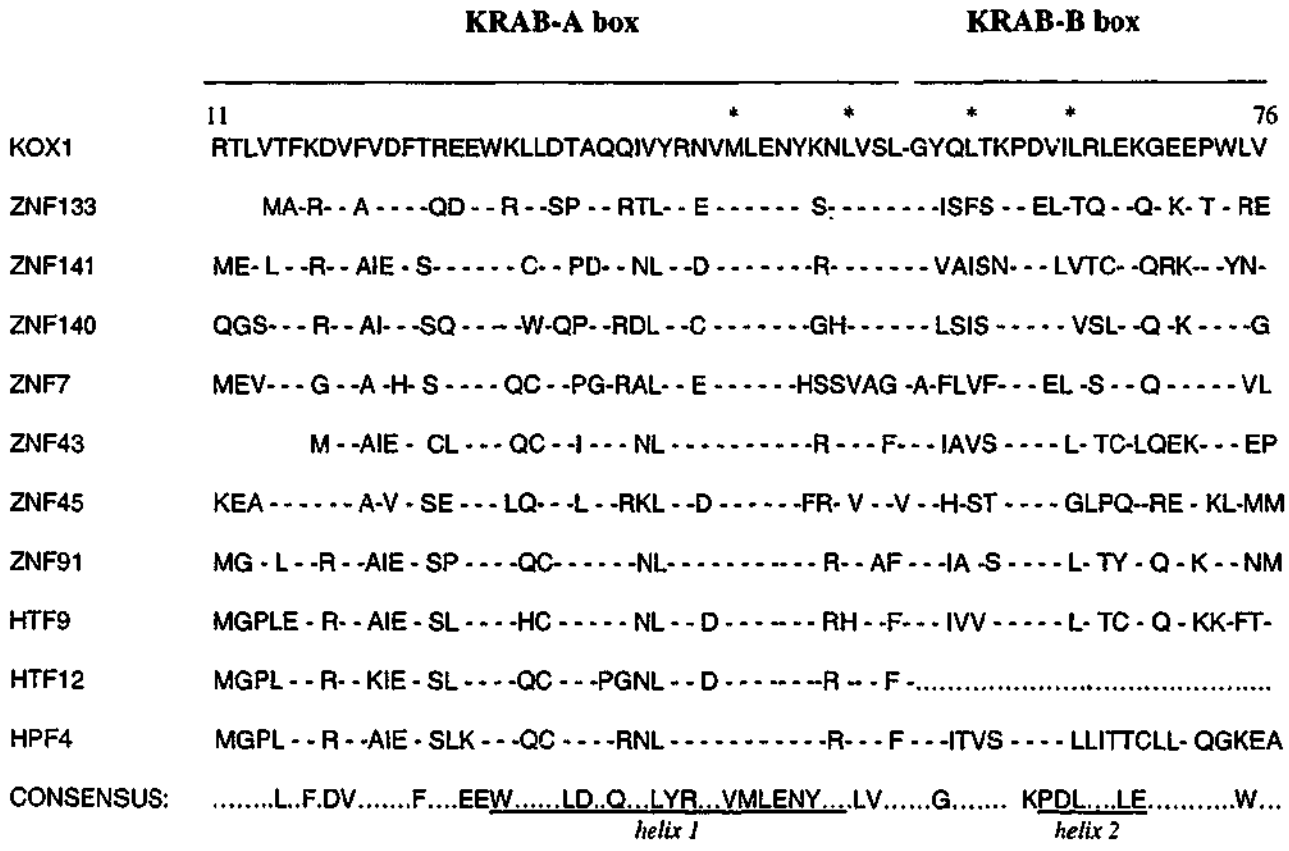


Fig. 1.9 Alignment of amino acid sequences of KRAB domains. The KRAB domains of 10 independent zinc finger gene products are aligned with the KOX1 sequence and the KRAB consensus. A dash indicates to be corresponding residue in KOX1; a period indicates a gap introduced to facilitate alignment (Adapted from Margolin *et al*, 1994; *Proc. Natl. Acad. Sci. USA.* 91:4509-4513).

1.6-3.3 Putative Mechanisms of Transcriptional Suppression by KRAB-DNA Binding Domain Fusion Protein

The molecular mechanisms by which the KRAB domain exerts its silencing function have not yet been identified. However, according to other eukaryotic repressors which exert transcriptional silencing, there may be three different ways: (I) by steric hindrance mechanisms similar to bacterial repressors which exert silencing effect (Brand *et al*, 1985); (ii) by specifically interaction with the RNA polymerase II basal or regulatory transcription machinery thereby switching off the transcription activity (Hanna-Rose and Hansen, 1996); (iii) and by inducing changes in the state of histone activation and/or recruiting proteins that participate in the remodeling of chromatin structure which leads to transcriptional repression (Kingston *et al*, 1996). In eukaryotes, the KRAB repression domain is believed to act by either directly inhibiting the transcriptional machinery and/or by altering the chromatin structure. These actions are mediated by the related intermediatory factors, TIF1α and TIF1β (also named KAP-1/KRIP-1), which interact with KRAB (Friedman *et al*, 1996; Kim *et al*, 1996; Le Douarin *et al*, 1996; Moosmann *et al*, 1996).

1.6-3.4 Co-factors of KRAB Zinc Finger Protein

KAP-1/KRIP-1 have no DNA-binding activity of their own and interact directly with the KRAB domain but not with KRAB mutants that do not repress transcription *in vivo* (Margolin *et al*, 1994; Friedman *et al*, 1996; Peng *et al*, 2000a). Thus, they might constitute corepressors for the large family of KRAB zinc-finger proteins. KAP-1 is a 97 kDa nuclear phosphoprotein that possesses several signature motifs, including a RING finger, B boxes, and a coiled-coil region (RBCC), which collectively form an integrated domain that is both necessary and sufficient to directly interact with the KRAB domain (Fig.1.10) (Friedman *et al*, 1996; Peng *et al*, 2000a, b). The carboxyl terminus of KAP-1 includes a PHD (plant homeodomain) finger and a bromodomain. All members of this protein family have been shown to repress transcription when tethered to DNA using a heterologous DNA-binding domain, and mutations in either domain weaken this repression activity (Peng *et al*, 2000a, b). The mechanisms by which they repress transcription are currently being defined. These studies suggest that

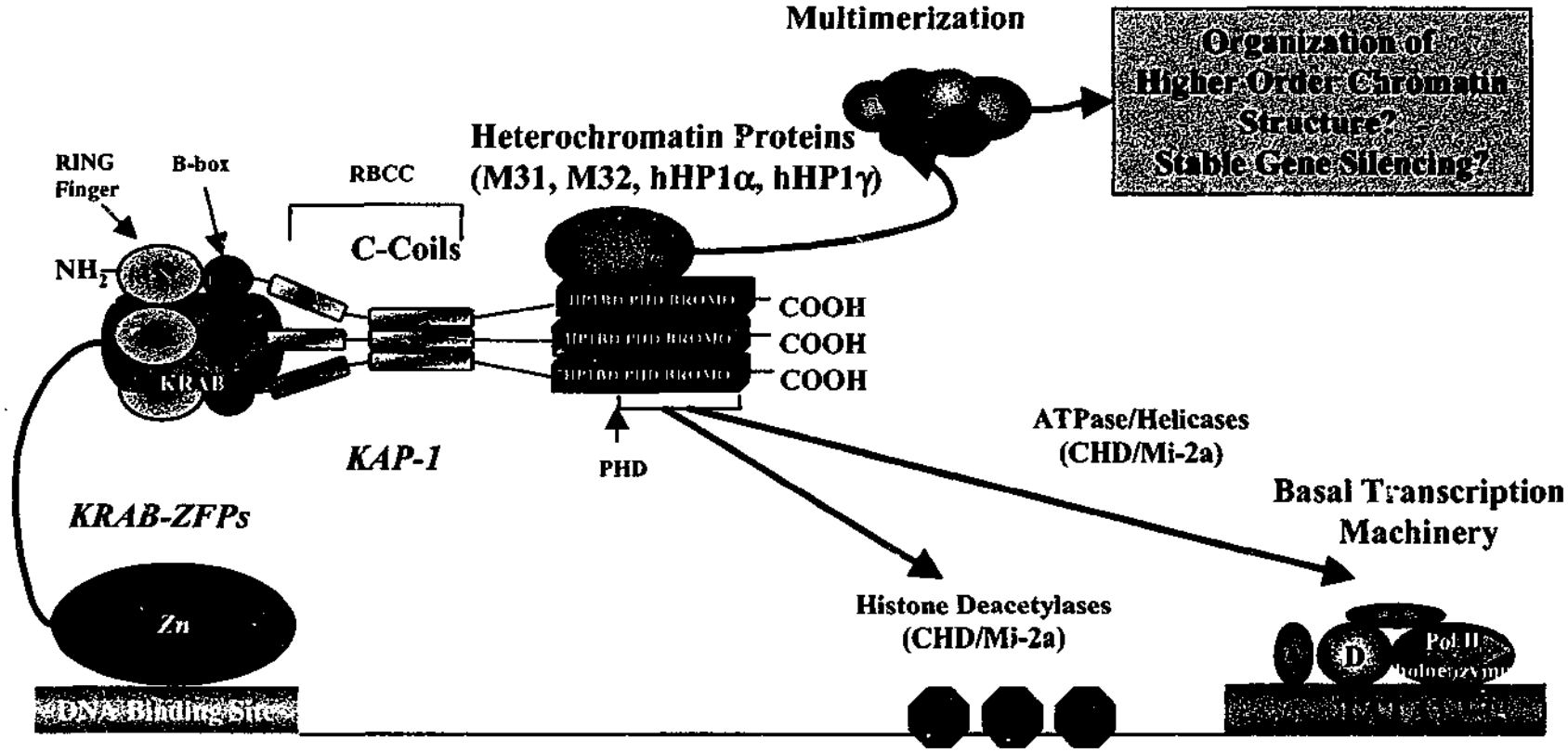


Fig.1.10 Schematic diagram showing protein-protein interactions identified in the KRAB-ZEP-KAP-1 repression pathway. KRAB-ZEP family of transcriptional repressors function in part as sequence-specific DNA-binding proteins which recruit the KAP-1 corepressor target gene. This interaction is dependent on the RBCC domain of KAP-1. Together, the HP1BD, PHD, and the bromodomain comprise the surfaces which mediate gene silencing via interaction with the indicated potential partners such as HP1 (Lechner *et al*, 2000) and Mi-2a (Schultz *et al*, 2001) which may mediate the assembly of stable, higher-order silenced domains and regulate chromatic dynamics in gene silencing in the eukaryotic nucleus. (Adapted from Ryan *et al*, 1999; *Mol Cell Biol* 19:4366-4378)

the KRAB-KAP-1 complex may mediate repression in part via the maintenance or initiation of heterochromatic chromosomal environments (Fig.1.10) (Ryan *et al*, 1999). The alteration of chromatin structure on promoter silences the transcriptional activity. Therefore, KAP-1, the co-factor of KRAB appears to be important in KRAB domain-mediated repression because of the following evidences: (i) the KRAB domain does not repress in cells that lack KAP-1; (ii) overexpression of KAP-1 enhances KRAB-mediated repression; (iii) and mutations at the KRAB-A module affect KAP-binding and this abolishes repression (Peng *et al*, 2000a). Furthermore, a direct interaction between KAP-1 and the nonhistone chromosomal protein, heterochromatin protein 1 (HP1) has also been shown to be important in KRAB-mediated suppression (Fig.1.10) (Le Douarin *et al*, 1996; Lechner *et al*, 2000). Another co-factor, Mi-2a which possesses and ATPase activities, interacts with KAP-1 and is also implicated in KRAB-mediated suppression (Fig.1.10) (Schultz *et al*, 2001). All these data suggest that the KRAB-KAP-1 complex may mediate repression by regulation of chromatic dynamics which results in transcriptional gene silencing.

1.7 Summary and Perspective

The Ewing family of tumours (ESFT) is a subgroup of small-round-cell tumours which shares a common neural histogenesis and tumour genetics. It is the second most common malignant bone tumour of children and adolescents. The annual incidence is 2.7 cases per million children and this tumour occurs mostly in Caucasians (Li *et al*, 1980; Fraumeni *et al*, 1981; Gurney *et al*, 1996). The precise aetiology of ESFT is unknown and it appears to be associated with neither radiation exposure nor any cancer family syndromes (Yamamota *et al*, 1960; Tucker *et al*, 1987). The treatments nowadays are surgery, chemotherapy and radiotherapy which can rescue almost two thirds of benign ESFT. However, the remission rate in metastatic ESFT is still low. In addition, the minimal residual disease of this tumour also causes difficulties in treatment of this tumour upon relapse. The diagnosis and prognosis for this tumour is difficult as this tumour family lacks specific phenotypic markers. Therefore, the identification of cellular markers for prognosis and new therapeutic avenues are important for this tumour.

By molecular genetic analysis, at least 90% of cases of ES/PNET have a clonal translocation seen in malignant cells. These translocations give a good marker for ES/PNET. Approximately 85% of translocations involves chromosomes 11 and 22: t(11;22)(q24;q12) (Wang *et al*, 1986; Turc-Carel *et al*, 1986). Another 55-10% of tumours contain an alternative rearrangement juxtaposing chromosome 21 and 22: t(21;22)(q21;q12) (Zucman *et al*, 1993; Sorensen *et al*, 1994). Molecular cloning and characterization of these translocation breakpoints revealed that a novel RNA binding protein of TET subfamily, *EWS*, at chromosome 22 fuses to one member of *ETS* transcription factor family; *FLI-1* at chromosome 11 or *ERG* at chromosome 21 (Delattre *et al*, 1992; Srensen *et al*, 1994).

The *EWS* gene is expressed ubiquitously and is involved in other translocations which occur in other tumours such as DSRCT, MMSP, Myoid Liposarcoma and Extraskeletal Myxoid Chondrosarcoma (Gerald *et al*, 1995; Zucman *et al*, 1993; Ron *et al*, 1992; Labelle *et al*, 1999). These reports indicated that there are some fragile points in some particular introns of *EWS* which facilitate the translocations in numbers of tumour. Sequence analysis of these breakpoints found that the junctions were flanked by various oligomers, *Alu* repeats and eukaryotic topoisomerase II cleavage sites (Obata *et al*, 1999). The size of Intron 6, particularly, of *EWS* involved most common translocation breakpoint region in ES/PNET, was different between white and black people (Zucman *et al*, 1997). The longer size of intron 6 of *EWS* in white causes genome plasticity and may give some insight into the racial difference of this tumour incidence (Zucman *et al*, 1999).

EWS/FLI-1 fusion protein has been suggested to have a key role in the pathogenesis in ES/PNET (Zucman *et al*, 1993; Soresen *et al*, 1994). In this fusion protein, the N-terminal of EWS fuses to C-terminal FLI-1 which increases the transactivation potential of wild-type FLI-1 forms an aberrant transcription factor (Delattre *et al*, 1992; May *et al*, 1993b). Human EWS/FLI-1 has been shown to transform NIH3T3 cells while deletion of either EWS or the EDB domain of EWS/FLI-1 completely abrogates the ability of tumcurigenicity on NIH3T3 cells. This suggests that EWS/FLI-1 does not act by interfering with either normal cellular *EWS* or *FLI-1* genes. In all ES/PNET translocation cases, only members of the ERG (FLI-1, ERG) and PEA3 (ETV1, E1AF) subclasses of the ETS family of transcription factors which share high homology in

EDB domain are rearranged (Fig.1.7). This may indicate that the conserved DNA binding domain of this subfamily provides the specific gene activity which gains stronger transactivational ability from EWS and that results in dysregulation of a subset of primary targets which in turn regulate other downstream oncogenes involved in tumourigenesis in ES/PNET. By RNA differentiation assay, genes such as *c-myc*, *stromelysin –1*, *Manic Fringe*, *mE2C* and *EAT2* etc. have been shown to be activated in Ewing's sarcoma (Bailly et al, 1994; Braun et al, 1995; Thompson et al, 1996; May et al, 1997; Arvand et al, 1998). They are upregulated by EWS/FLI-1 in ES/PNET cells and associated with tumourigenesis. However, there are no strong evidences to show these genes are direct targets of EWS/FLI-1. Therefore, examining the primary target genes of EWS/FLI-1 will be extremely useful to develop new treatment methods and understand the pathological mechanisms. All data about the role of EWS/FLI-1 in pathogenesis of ES/PNET come from *in vitro* experiments. The deeper insight on the role of this fusion protein and the aetiology of ES/PNET is necessary. A mouse model will be necessary to give *in vivo* evidence on the role of EWS/FLI-1.

Although there are many currently treatments to improve the remission rate of ESFT, better therapeutic methods are required. The discovery of novel chemotherapeutic drugs and gene therapy will be a good avenue for the therapy regimens of this tumour. There are many approaches to inhibit transformed phenotypes of ES/PNET such as using antisense *EWS/FLI-1* mRNA (Ouchida *et al*, 1995; Kovar *et al*, 1996) and antisense oligonucleotides of *EWS/FLI-1* transcripts (*Tanaka et al*, 1997; Toretsky *et al*, 1997; Wang *et al*, 1999). All these approaches are based on the reduction of *EWS/FLI-1* mRNA and protein levels in ES/PNET cells and so abrogate the oncogenic pathways in ESFT cells. Another potential approach for therapy of ES/PNET is the use of the DNA-dependent potent transcriptional repression module, KRAB (Margolin *et al*, 1994; Vissing *et al*, 1995). The KRAB/DNA-binding-domain fusion proteins have been shown to efficiently suppress promoter-reporter constructs of *erbB-2/HER-2*, *HIV-1*, and *estrogen receptor* promoters in a DNA binding specific manner (Beerli *et al*, 1998; Herchenroder *et al*, 1999; Ma *et al*, 1999; de Haan *et al*, 2000). Additionally, when KRAB was engineered to PAX3 domain to be a PAX3-KRAB transcriptional repressor, it has been shown to efficiently inhibit the transformed phenotype of alveolar rhabdmyosarcoma tumours (ARMS) (Fredericks *et al*, 2000).

1.8 Aims of this Study

From the above literatures review, the EWS/FLI-1 or other EWS-ETS fusion proteins are believed to play a key role in the pathogenesis of ES/PNET. To further understand the oncogenic mechanisms of these fusion proteins and aetiology of ES/PNET, a mouse model mimicking human ES/PNET will be a fantastic tool to study. In addition, such a model will be useful for developing new therapeutic strategies. However, before generating this mouse model, the tumourigenecity of murine and human EWS/FLI-1 fusion proteins and the mouse *EWS* genomic structure and expression patterns need to be examined and compared with the human *EWS*. Moreover, potential avenues (e.g. KRAB/FLI-1) for inhibiting the action of this potent transcription factor (EWS/FLI-1) need to be first assessed *in vitro*. Therefore, the specific aims and directions of this study include:

1. To generate a mouse *EWS/Fli-1* fusion gene and compare its putative tumorigenicity with that of the human *EWS/FLI-1* fusion gene.
2. To investigate the suppression effects of an engineered KRAB/FLI-1 repressor on the EWS/Fli-1 transformed phenotype.
3. To investigate a novel human ETS member, *ER99* which expressed in ES/PNET and upregulated by EWS/FLI-1.
4. To characterize the mouse *EWS* gene and generate *EWS* gene knockout and *EWS/Fli-1* fusion gene knock-in targeting constructs.

Chapter 2
Materials and Methods

2.1 Materials

Analytical and general grade inorganic or organic chemicals were purchased from a number of local and international suppliers. Unless indicated, otherwise, all laboratory reagents were analytical grade and supplied from Ajax Chemicals, Australia; BDH Chemicals, UK; Bio-Rad Laboratories, USA; Boehringer Mannheim, Germany; Progen Industries, USA; Promega Corporation, USA; or Sigma Aldrich, USA.

The radioisotopes such as (α-^{32}P) dCTP, (γ-^{32}P) ATP (10mCi/ml) were purchased from Amersham International Pty Ltd. The restriction endonucleases and other enzymes except where specially mentioned were purchased from Promega Corporation, USA; Boehringer Mannheim, Germany; GIBCO BRL, UK; New England Biolabs, USA.

2.1-1 List of Solutions, Suppliers, and Oligonucleotides

Appendix 1 List of Media

Appendix 2 List of Buffers and Solutions

Appendix 3 List of Suppliers

Appendix 4 Positions and Sequences of Oligonucleotides

2.1-2 Bacterial Strains

Strain	Genotype	Used For	Supplier
JM109	*end*A1, *rec*A1, *syr*A96, *thi*-1, *Hsd*R17, (rk-, mk+), *rel*A1, *Sup*E44, λ-△ (*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*I^{q}Z△M15]	Plasmid transformation	Promega
XL1-Blue	recA1, endA1, gyrA86, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac*[F', *pro*AB, *lac*I^{q}Z△M15, Tn10, (tet^r), Amy, cam^r]	Plasmid transformation Blue/white selection	Stratgene

2.1-3 Eukaryotic Cell Lines

NIH3T3: Murine embryo fibroblast cell line (ATCC CRL-1658). This cell line, established from NIH Swiss mouse embryo culture is highly contact inhibited and therefore is often used for transformation assay (Anderson *et al*, 1978; Copeland *et al*, 1979).

SK-N-MC: A human neuroblastoma cell line with type I EWS/FLI-1 fusion created from chromosomal translocation t(11; 22)(q24; q12). It was obtained from American Type Culture Collection (ATCC, Cat. no. HTB-10), Manassas, VA 20110-2209, USA.

ZR-75-1: A human breast cancer epithelial cell line. It was obtained from ATCC (Cat. no. CRL-1500).

T-47D: A human breast cancer epithelial cell line. It was obtained from ATCC (Cat. no. HTB-133).

MCF7: A human breast cancer epithelial cell line. It was obtained from ATCC (Cat. no. HTB-22).

MDA-MB-435: A human breast cancer epithelial cell line with spindle shaped strain. It was obtained from ATCC (Cat. no. HTB-129).

MDA-MB-453: A human breast cancer epithelial cell line. It was obtained from ATCC (Cat. no. HTB-131).

BML1 and BMB1: Two mouse breast cancer cell lines from C57BL mice which were obtained from Dr. M. Tavaria (Peter MacCallum Cancer Institute, Melbourne, Australia).

Embryonic Stem (ES) Cell: The mouse MPI ES cell line was provide by Millennium Pharmaceuticals, Cambridge, MA 02139, USA. This cell line was developed from 129S6/SvErTac the mouse strain. It gives to white-bellied agouti in chimaeric mice.

All cell lines were maintained and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) or RPMI 1640 (Gibco-BRL) medium supplemented with 10% foetal calf serum (FCS), 1% penicillin/streptomycin (Gibco-BRL), and cultured in a humidified incubator in 5% CO_2 at 37^0C.

2.1-4 Mouse Strain

BALB/c nu/nu (nude) mice: Female *BALB/c* mice at 5-7 weeks, carrying the *nu/nu* mutation were obtained from the Animal Resource Centre (ARC), Perth. Nude mice were maintained under SPF condition in the Monash Institute of Reproduction and Development animal house. All handling was reduced to a minimum and was carried out in surgical gowns, masks, gloves and overshoes which had been sterilized by UV illumination. Sterile water and food was provided *ad libitum*, and the mice were kept at a constant 25^0C on a 24 hours day/night cycle.

2.1-5 Antibodies

All the following antibodies were obtained from Santa Cruz Biotechnology, Inc, USA.

Fli-1 (C-19) (cat. no. sc-356): Affinity-purified rabbit polyclonal antibody raised against a peptide located at the carboxyl terminus of mouse Fli-1 (differs from the corresponding human sequence by a single amino acid).

c-myc (N-262) (cat. no. sc-764): Affinity-purified rabbit polyclonal antibody raised against amino acids 1-262 of human c-myc (includes the transcriptional activation region, but not the bHLH-Zip protein-protein interaction domain).

MMP-3 (C-19) (cat. no. sc-6839): Affinity-purified goat polyclonal antibody raised against a peptide from the carboxyl terminus of human MMP-3 (matrix metalloproteinase-3) (differs from the corresponding mouse sequence by two amino acids).

α-tubulin (TU-02) (sc-8035): Affinity-purified mouse polyclonal antibody raised against full-length porcine α tubulin.

2.2 DNA Manipulations

2.2-1 Restriction Enzyme Digestions

Restriction enzyme digestions were performed on purified plasmid, bacteriophage or genomic DNA, according to the recommendations of the manufacturer (Promega). For example, 1 μg plasmid DNA was digested with 20 units restriction enzyme (s) and the recommended 1 x restriction enzyme buffer in a total volume of 20-40 μl. The digestion was allowed to proceed at 37^0C for 1 hour (plasmid DNA) or overnight (for bacteriophage or genomic DNA). The reaction result was analysed by agarose gel electrophoresis (Section 2.2-2).

2.2-2 Agarose Gel Electrophoresis (DNA Electrophoresis)

Agarose gels of varying concentrations (0.5% to 2.0%) were prepared by boiling powdered agarose (Progen) in 1 x TAE. When the solution had cooled to approximately 50^0C, 0.5 μg/ml of ethidium bromide (EtBr) was added and the unit was poured into a gel cast and allowed to set. The digested DNA samples containing 1 x DNA loading dye were loaded into wells and electrophoresed at 30 – 90 V in 1 x TAE running buffer in a horizontal agarose gel apparatus (BIO-RAD: mini sub). The DNA was visualized by placing the gel on a short-wave UV transilluminator and photographed. Bacteriophage DNA digestions were heated at 65^0C for 10 minutes prior to loading into wells to separate the cohesive ends of the bacteriophage lambda (λ) arms.

2.2-3 Recovery of DNA Fragments from Normal Agarose Gels

After electrophoresis on agarose gel, the minimal gel slices containing the required DNA bands were cut under UV illumination. DNA fragments were recovered using Gel Extraction Kit QIAE II (QIAGEN) according to the manufacturer's instructions.

2.2-4 Blunt Ending

Restriction enzyme digested DNA fragments and vector DNA which had non-compatible cohesive termini were blunt-ended before ligation. DNA with 5' overhang sticky ends was supplemented with 10 μl Klenow 10 x buffer, 1 μl 10mM dNTPs (dATP, dTTP, dCTP, dGTP), 10 units Klenow enzyme (Promega) and with MQH_2O in a final volume of 100 μl. The mixture was incubated at room temperature for 15 minutes and the reaction was stopped by incubating at 65^0C for 15 minutes.

For DNA with 3' overhang sticky end product, the DNA was supplemented with 10 μl Klenow 10 x buffer or DNA polymerase buffer, 25 units Klenow enzyme or T4 polymerase (Promega) and with MQH_2O in a final volume of 100 μl. The mixture was

incubated at room temperature for 15 minutes and the reaction was stopped by incubated at 65^0C for 15 minutes.

For 5' and 3' overhang sticky ends, the 3' overhang reaction was first performed, and 1 µl of dNTPs was added next to fill the 5' overhang. The enzyme was inactivated at 65^0C for 15 minutes. The required DNA fragments were purified from excess deoxynucleotides or other DNA fragment by-products by agarose gel electrophoresis followed by Gel Extraction Kit (Qiagen) (see Section 2.2-3).

2.2-5 Dephosphorylation

To reduce the recircularization of the vector DNA during ligation, it was treated with calf intestinal alkaline phosphatase (CIAP) under the conditions recommended by the manufacturer (Promega) to remove 5'-phosphate residues. In case of the blunt end cloning, the following components were added directly to the blunted DNA: appropriate volume of CIAP 10 x buffer, 0.1U (1µl) CIAP (Promega) and MQH_2O. For protruding 5'-termini dephosphorylation, the mixture was incubated for 30 minutes at 37^0C. Another 0.1U CIAP (1 µl) was then added and incubated for an additional 30 minutes at 37^0C. For recessed 5'-termini or blunt end dephosphorylation, the mixture was incubated for 15 minutes at 37^0C, then for 15 minutes at 56^0C. Another 0.1U CIAP (1µl) was added and the incubations were repeated at both temperatures. To stop the reaction, 2.0 µl of 0.5M EDTA was added and the unit was heated for 20 minutes at 65^0C. The dephosphorylated DNA was then purified as described in Section 2.2-3.

2.2-6 DNA Ligations

Phosphatased, linearized vector was ligated with a DNA fragment under the conditions recommended by the manufacturer (Promega). The amount of insert DNA to be used was calculated according to the following formula:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

A 1:1 and 1:3 vector : insert ratios were used by setting up the following ligation reaction.

	1:1	1:3
Vector	**100μg**	**100μg**
Insert	**17μg**	**51μg**
T4 DNA Ligase	**1U**	**1U**
Ligase 10 x Buffer	**1 μl**	**1 μl**
MQH_2O	**to a final volume of 10 μl**	

The ligation reaction was allowed to proceed overnight at room temperature for blunt end ligation, or overnight at 4^0C for cohesive end ligation.

2.2-7 *Taq* Polymerase Chain Reaction (PCR)

Some DNA fragments used for cloning procedures were generated by PCR which was carried out on genomic or cDNA templates. The reaction volume consisted of 5 μl of plasmid cDNA(10 pg/ μl) or genomic DNA (20 ng/ μl) mixed with 5 μl 10 x *Taq* DNA polymerase buffer (Promega), 125 μM dNTP's, 1.5 mM $MgCl_2$, 50 pM of each oligonucleotide primer and 0.5 units of *Taq* DNA polymerase (Promega) made up to a final volume of 50 μl with MQH_2O. A three-steps temperature cycle was repeated 35 times after 5 minutes 94^0C denaturing initiation step using the Gene Amp PCR System 2400 (Perkin Elmer): for example, 30 seconds at 94^0C (denaturing), 30 seconds 55^0C (annealing: dependent on primer melting temperature) and 1 minute 72^0C (polymerization: dependent on the length of the amplified fragment). PCR products were analysed by agarose gel electrophoresis, as described in Section 2.2-2, recovered from agarose gels for ligation, as described in Section 2.2-3, and cloned into pGEMT vector by using TA Cloning™ System.

2.2-8 *Elongase* Polymerase Chain Reaction (PCR)

To amplify a long fragment (> 2kb) without point mutations, *Elongase* PCR was used to amplify *EWS* genomic fragments and murine *EWS* cDNA in this study. The enzyme, *Elongase* (Boehringer Mannheim Biochem), contains a mixture of *Taq* and *Pyrococcus*

species GB-D thermostable DNA polymerase, an enzyme with 3'→5' exonuclease activity to prevent point mutations. A 50 µl volume of PCR reaction consisted of 5 µl genomic DNA (50 ng/µl), 3 µl buffer A (3 mM $MgCl_2$), 7 µl buffer B, 125 µM dNTPs, 50 pM of each oligonucleotide primer and 5 units of *Elongase* enzyme (1µl) (Boehringer Mannheim Biochem) made up to a final volume of 50 µl with MQH_2O. A three-steps temperature cycle was repeated 35 times after 5 minutes 94^0C denaturing initiation step using the Gene Amp PCR System 2400 (Perkin Elmer): for example, 30 seconds at 94^0C (denaturing), 30 seconds 55^0C (annealing: dependent on primer melting temperature) and 5 minutes 68^0C (polymerization: dependent on the length of amplified fragment, 1 minute equals to 1 kb). PCR products were analysed by agarose gel electrophoresis, as described in Section 2.2-2, and recovered from agarose gels for ligation, as described in Section 2.2-3. The purified PCR product was added 5'-A overhang by supplementation of dATP, 5 µl 10 x *Taq* DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, and 0.5 units of *Taq* DNA polymerase (Promega) made up to a final volume of 50 µl with MQH_2O. The reaction was carried at 72^0C for 15 minutes and cooled down to 4^0C. The overhang-A PCR product was gel purification, recovered as described in Sections 2.2-2 and 2.2-3, and cloned into pGEM-T vector by using TA Cloning™ System.

2.2-9 Direct Cloning of PCR Products Using TA Cloning™ System

PCR-generated DNA products which have 5'-A overhangs were ligated directly into pGEM-T vector which has 3'-T overhangs, according to the manufacturer's instructions (Promega). The ligation mixture containing 1µl pGEM-T vector (25 ng), 1 µl Ligase 10 x buffer, 1 units T4 DNA Ligase (1 µl) and insert DNA (DNA to vector ratio was based on the formula in Section 2.2-6) which was made up to a final volume of 10 µl by MQH_2O. The ligation mix was incubated overnight at room temperature or 4^0C.

2.2-10 Bacterial Transformation

The ligated plasmid was transformed into *E.coli* JM109 competent bacteria under the conditions recommended by the manufacturer (Promega). A 200 µl aliquot of *E.coli* JM109 competent cells (stored at -70^0C) was thawed on ice. The DNA ligation mixture

(1-2 µl; 10-20 µg) was incubated with 50 µl of competent cells on ice for 30 minutes heat-shocked at 42^{0}C for 45 seconds, and then cooled immediately on ice for 2 minutes. The transformed bacteria were allowed to propagate in 400 µl of SOC medium for 45 minutes in a 37^{0}C shaking incubator. Aliquots of transformation mix (150 µl and 250 µl) were plated onto the LB (Luria-Bertani) agar plates supplemented with 50 µg/ml ampicillin (amp) overnight at 37^{0}C.

2.2-11 Blue/White Selection of Recombinant Clones

When using vectors containing multiple cloning sites within the *β-galactosidase* gene (*lacZ*) (such as pGEM-T and pBluescript KS+ vectors), selection by blue/white colour was used. LB/Amp (50 µg/ml) agar plates were coated with 20 µl of 50mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside in N,N' dimethylformamide), 100 µl of 100 mM isopropylthio–β-D-galactoside (IPTG) and allowed to dry for 30 minutes at 37^{0}C prior to plating transformed bacteria. After incubation overnight at 37^{0}C, white-colored colonies signifying recombination were selected for plasmid DNA mini-prep according to Section 2.2-12.

2.2-12 Isolation of Plasmid DNA

Putative recombinant bacterial colonies of *E.coli* arising from transformation by ligation products, were propagated in liquid media and their plasmid DNA extracted by min-prep procedures. Diagnostic restriction enzyme digestions were used to screen these clones for proper vector-insert positions and orientation. The correct plasmids were then expanded by maxi-prep procedures.

Single Tube Miniprep Method:

Colonies were picked from a master plate and transferred into 1.5 ml-eppendorf tubes with 300 µl Terrific Broth and appropriate antibiotics. Tubes were incubated overnight at 37^{0}C with shaking, then spun at 13,000 rpm for 1 minute to remove the cell pellet. It was resuspended in 40 µl STT containing 10 mg/ml Lysozyme and incubated at 95-

100^{0}C for 1 minute. It was then spun at 13,000 rpm for 5 minutes and 20 µl of the supernatant was used for restriction enzyme digestions and analysis on agarose gel.

Maxi Plasmid Preparation:

A modified alkaline lysis/caesium chloride method (Sambrook *et al*, 1997) was used for large-scale plasmid DNA preparations. A single bacterial colony containing a required DNA insert was inoculated into 250 mls Terrific Broth (TB) supplemented with 50 µg/ml ampicillin overnight in a 37^{0}C shaking incubator. The bacterial culture was harvested by spinning at 5,000 rpm for 10 minutes at 4^{0}C in a JA10 rotor at J2-21M/E Centrifuge (Beckman) and the bacterial cell pellet was resuspended in Solution I supplemented with 2 mg/ml of lysozyme which was incubated at room temperature for 8 minutes. The cell suspension was mixed with 40 mls of freshly prepared Solution II and incubated at room temperature for 10 minutes. Bacterial cell debris, genomic DNA and proteins were precipitated by adding of 30 mls solution III and was left on ice for 20 minutes. The precipitate was removed by centrifugation at 10,000 rpm for 15 minutes at 4^{0}C, and the supernatant was strained through gauze. Plasmid DNA was precipitated by addition of 50 mls of isopropanol and pelleted by centrifugation at 10,000 rpm for 20 minutes at room temperature. The DNA pellet was rinsed with cold 70% v/v ethanol, inverted to air-dry and resuspended in 3.5 mls of MQH$_2$O of 1 g/ml Caesium Chloride (CsCl) and 20 µl of 10 mg/ml EtBr. The plasmid DNA solution was transferred to a heat sealable TL100 ultracentrifuge tube (Beckman) and centrifuged in TL-100 Ultracentrifuge (Beckman) for 20 hours at 100,000 rpm at 20^{0}C. The supercoiled plasmid band was collected with a syringe and 18 gauge needle into a fresh tube. The EtBr was removed by using water saturated butanol extractions until all EtBr was removed. Plasmid DNA was precipitated by addition of 5 mls isopropanol and pelleted by centrifugation at 5,000 rpm for 10 minutes. The DNA pellet was washed by cold 70% ethanol, air-dried and resuspended in 2 mls of TE. The concentration of DNA was measured by UV/VIS Spectrometer Lambda Bio20 (Perkin Elmer).

2.2-13 DNA Sequencing

Sequencing of plasmid DNA was performed in-house by the Sequencing Facility using the *Taq* DyeDeoxy terminator Sequencing kit on an Applied Biosystems automatic

DNA sequencer model 373A. Primers used included gene-specific primers and commercially available T3, T7 and Sp6 sequencing primers (Promega). The inserts were sequenced at least once in each direction.

2.2-14 Computer Analysis

Analysis and alignment of nucleotide and protein sequences was performed using the sequence analysis software package by the Genetics Computer Group (GCG; Altschul *et al*, 1990,1997), BLASTN, BLASTP and TASTA- homology searches against the GenBank, EMBL and SWISS-PROT nucleotide and protein databases.

2.2-15 Random Primed DNA Labeling

Double stranded DNA (dsDNA) was labelled with [α-^{32}P] dCTP (10 mCi/ml; Amersham) using a random primed DNA labeling kit, according to the manufacturer's instructions (Boehringer Mannheim Biochem). After incubation for 30 minutes at 37^{0}C, unincorporated [α-^{32}P] dCTP was removed by column chromatography on a 2 mls column of G-50 Sephadex beads (Amersham Pharmacia) by elution in TE buffer. The activity of the labelled DNA was determined using a β-scintillation counter as counts per min (cpm) in the presence of 5 mls hydrofluor scintillation fluid (BDH Biochemicals). A total of 2 x 10^6 cpm labelled DNA per ml of hybridisation buffer was denatured prior to hybridisation by heating at 95^{0}C for 5 minutes.

2.2-16 End-labeling of Oligonucleotides

Single-stranded oligonucleotides (~20pmol) were labelled with 4 μl of [γ-^{32}P] dATP (10 μCi/μl; Amersham) by incubation with 1μl of T4 polynucleotide kinase 10 x buffer (Promega), 3 μl of MQH$_2$O and 1 μl of T4 polynucleotide kinase (Promega) at 37^{0}C for 30 minutes. The activity of T4 polynucleotide kinase was inactivated by incubation at 90^{0}C for 2 minutes. The entire reaction mixture was used for hybridization.

2.2-17 Southern Blot Analysis

DNA was transferred onto a Hybond-N nylon membrane (Amersham) by capillary elution with 0.4% NaOH buffer. If the DNA of interest was larger than 15 kb, the gel was treated with 0.2 M HCl for 20 minutes and washed by 2 x SSC before transferring. Following DNA transfer to the Hybridization Transfer Membrane Gene Screen Plus (NEN™ Life Science Products), the DNA was fixed on the membrane by air-drying or baked in a 60^{0}C incubator until dry. The filter was prehybridized for 2-3 hours at 65^{0}C in 5 x SSPE, 5 x Denhardt's solution, 0.5% (w/v) SDS and 20 μg/ml of salmon sperm DNA, and then hybridized in the same solution with labelled DNA probe for 16 hours at 65^{0}C, while 50^{0}C was used for labelled oligonucleotide probes. The filter was washed at increasing stringencies depending on the sequence identity and length of the probe used. For most homologous cDNA probes, the filter was washed in 2 x, 1x and 0.2 x SSPE containing 0.1% SDS at 65^{0}C for 10 minutes each, and exposed on a Kodak X-AR film with intensifying screens at –70^{0}C or on a Fuji phosphoimage screen using a Fuji BAS 1000 phosphoimage analyzer at room temperature.

2.2-18 Photography, Autoradiography and Phosphoimaging

Agarose gels were photographed using Bio-Rad Gel Doc 1000 ultraviolet box with camera. The photo was processed through a Video Copy Processor (Mitsubishi P90) and thermal paper (Mitsubishi). Autoradiography was performed using Kodak X-AR5 film in lightproof cassettes with intensifying screens, usually at –80^{0}C for 16 hours. Radiolabelled filters were exposed to phosphoimage screens for 30 minutes to 16 hours and scanned by a Fuji Bas 1000 phosphoimager. Quantitation was performed by BAS 1000 software.

2.3 Bacteriophage λ Manipulations

2.3-1 Library Screening

cDNA and genomic library screenings were performed to isolate cDNA and genomic clones, respectively. To isolate murine *EWS* genomic clones, the partial length of

human *EWS* cDNA (650 bp cut from human Type I *EWS/FLI-1* fusion gene) was used as a probe to screen a 129SvJ mouse genomic library in the bacteriophage λFIXII vector (Stratagene).

2.3-2 Preparation of Plating Bacteria for Bacteriophage λ

For the bacteriophage λFIXII (Stratagene), Y1090r⁻, XL-1 blue MRF' and XL-1 blue MRA (P2) strains of bacterial host cells were used to absorb. Host bacteria were grown in 50 mls NZYM containing 0.2% of maltose and 10 mM $MgSO_4$ overnight in a 37^0C shaking incubator, according to Sambrook *et al* (1989). The cell pellet was collected by spinning at 5,000 rpm for 10 minutes at room temperature in a MSE bench-top centrifuge, and resuspended in 5 mls of 0.01M $MgSO_4$. The cell suspension was stored at 4^0C for up to 2 weeks.

2.3-3 Plating Bacteriophage

To absorb the bacteriophage particles to the bacteria, 0.1 mls bacteriophage stock in SM were incubated with 0.1 mls of plating bacteria for 20 minutes at 37^0C according to Sambrook *et al* (1997). The phage mixture was mixed with 3 mls of 42^0C top NZYM agar, and poured onto a warm 9 cm NZYM bottom agar plates (9 mls of top agar for 15 cm plates). After cooling down to room temperature for 10 minutes, the plates were incubated for about 16 hours at 37^0C to obtain plaques.

2.3-4 Plaque Blotting

Bacteriophage were transferred onto circular Hybond-N nylon membranes (Amersham) and hybridised as described in Sambrook *et al* (1997). Circular nylon filters were placed on the bacterial plates containing bacteriophage plaques for 1 minute and 3 minutes (duplicate filter), allowing bacteriophage particles to transfer to the membrane. Orientation marks on the filter and plate were made by piercing the membrane with an ink filled syringe. The filters were then placed in a denaturing solution (1.5 M NaCl, 0.5

M NaOH) for 1 minute, a neutralizing solution (1.5 M NaCl, 0.5 M Tris-Cl [pH8.0]) for 5 minutes, rinsed in 2 x SSPE and air dried prior to hybridization.

2.3-5 Preparation of a Plaque Lysate Stock From a Single Plaque

To prepare the plate lysate stock, a single plaque was picked from the plate using a glass Pasteur pipette and placed in 1 ml of SM containing 20 µl of chloroform according to Sambrook *et al* (1997). After incubation of the mixture overnight at 4^0C, the titre of the stock solution was measured by serial dilutions with SM. To prepare a plate lysate of the phage, 10^5 pfu of the mixture were plated onto a 15 cm plate. Once the plaques had grown to the point where they were almost confluent, 15 mls of SM was added to the plate. The phage was eluted overnight at 4^0C. The SM was taken from the plate and 0.2 mls of chloroform was added, vortexed, and centrifuged at 5,000 rpm for 10 minutes at 4^0C in the MSE bench-top centrifuge. After recovery of the supernatant, 50 µl of chloroform was added to the plate lysate and stored at 4^0C. The titre of the lysate was determined by dilutions.

2.3-6 Large-scale Phage DNA Extraction

Phage DNA was prepared from a 500 mls culture of infected bacteria according to Sambrook *et al* (1997). A single colony was grown overnight at 37^0C in 10 mls of NZYM and 0.2% maltose in a shaking incubator. After inoculation of 5 mls of overnight culture into 500 mls of NZYM, the culture was incubated at 37^0C with shaking until the absorbance of the culture reached 0.5 at 600 nm (OD_{600}). The culture was inoculated with 10^{10} pfu of the bacteriophage λ and incubated at 37^0C with further shaking until cell lysis occurred. 10 mls of chloroform was added to the lysed culture and incubated for 10 minutes at 37^0C with shaking. After cooling, the lysed culture to room temperature, 500 µg of both pancreatic DNase I (Boehringer Mannheim Biochem) and RNase A were added, and incubated for 30 minutes at room temperature. 29.2 mls of NaCl to a final concentration of 1 M was added to the mixture and incubated for 1 hour on ice. After centrifugation 7,000 rpm for 10 minutes at 4^0C in a J2-21 M/E Beckman centrifuge, the supernatant was added to 50 g of polyethylene glycol (PEG)

8000 (Sigma) and incubated at room temperature until the PEG had dissolved by slow stirring. The bacteriophage particles were precipitated following 1 hour on ice and centrifugation at 7,000 rpm for 10 minutes at 4^0C. The precipitate was dissolved in 5 mls of SM, an equal volume of chloroform was added, and vortexed for 30 seconds. After centrifugation at 5,000 rpm for 15 minutes at 4^0C, 0.75 g of CsCl per ml of bacteriophage suspension was added to the aqueous phase. The bacteriophage suspension was transferred into two heat-sealable TL100 Beckman centrifuge tubes and centrifuged in a Beckman TL100 centrifuge at 100,000rpm overnight at 20^0C. The bacteriophage band was carefully removed using a syringe needle and transferred to a microtube. To remove CsCl, the purified bacteriophage solution was dialyzed twice at room temperature for 1 hour each against 2 litres of dialysis buffer. Following dialysis, 0.5 M EDTA (pH 8.0) was added to a final concentration of 20 mM, proteinase K to a final concentration of 50 µg/ml, and 10%SDS to a final concentration of 0.5%, and the bacteriophage proteins were digested for 1 hour at 56^0C. An equal volume of TE-saturated phenol:chloroform (50:50) was added to the extracted aqueous phase, vortexed, and re-extracted with a equal volume of chloroform. The bacteriophage DNA in the aqueous phase was precipitated by adding 3 M sodium acetate (pH 7.0) to a final concentration of 0.3 M and 2 volumes of 100% ethanol. After incubation at room temperature for 30 minutes, the DNA was pelleted by centrifugation at 12,000 rpm for 15 minutes at room temperature. The pellet was washed with 1ml of 70% ethanol, dried briefly in a vacuum desicator and resuspended in 50 µl of TE.

2.3-7 Dot Blot Screening

Dot blot was performed to characterize the murine *EWS* genomic fragments. 100 ng of bacteriophage DNA was blotted onto the Hybond-Nitrocellulose membrane (Amersham). The membrane was then denatured in 1.5 M NaCl/0.5M NaOH for five min and neutralized in 1.5 M NaCl/0.5M Tris-HCl (pH8.0) for 5 minutes. The procedure was repeated once. The DNA was crosslinked onto the membrane by exposing to UV for 5 minutes. The membrane was prehybridized in 3 mls of a solution containing 5 x SSC, 5 x Denhardt's, 1% SDS and 100 µg/ml denatured herring sperm DNA (Boehringer Mannheim) at 42^0C for 1-2 hours, and then hybridized in a rotating oven (Bartelt Instruments) with appropriate (γ-^{32}P) dATP labelled oligonucleotide in 3

mls of fresh solution at 42^{0}C overnight. Membrane was washed twice in 1 x SSC/0.1% SDS for 10 minutes at 42^{0}C, and then exposed to a Fuji phosphorimage screen and visualized using a phosphorimage analyzer (FLA-[illegible]; FUJIFILM, Tokyo, Japan).

2.4 RNA Manipulations

2.4-1 Total RNA Extraction

Total RNA was extracted from cell lines according to a modified method of Chomczynski and Sacchi (1987). Frozen cell pellet was homogenized in 10 mls Guanidinium thiocyanate homogenization buffer using an Ultra-Turrax T25 homogenizer. Ice-cold 0.1 volume of 2 M sodium acetate (pH 4.0), one volume of ice-cold MQH_2O-saturated phenol and 0.2 volumes of ice-cold chloroform : isoamyl alcohol (49:1) were added sequentially with vigorous shaking. After incubation on ice for 20 minutes, the mixture was centrifuged at 5,000 rpm (J2-21 M/E; Beckman) for 20 minutes at 4^{0}C and the aqueous phase removed. One volume of phenol and one volume of chloroform : isoamyl alcohol (49:1) was added and following shaking and centrifugation as above, the aqueous phase was removed. This procedure was repeated until the aqueous phase became free of particulate matter. Total RNA was precipitated by addition of an equal volume of isopropanol followed by incubation overnight at –20^{0}C. The RNA pellet was obtained by centrifugation at 5,000 rpm (J2-21 M/E; Beckman) at 4^{0}C for 15 minutes, and then washed twice with ice-cold 75% v/v ethanol, air-dried, resuspended in 50-100 µl of DEPC-treated MQH_2O and stored at –70^{0}C. The concentration and quality of the total RNA were determined by UV spectrophotometer at OD_{260nm} and OD_{280nm} (Lambda Bio20; Perkin Elmer). Pure preparations of RNA have OD_{260nm}/ OD_{280nm} values of approximately 2.0.

2.4-2 Poly A$^+$ RNA Extraction

The poly A$^+$ RNA was extracted from tissues or cells using Oligo-dT cellulose (Boehringer Mannheim). Frozen tissue or cell pellet was homogenized in 25 mls

extraction buffer and 300 µl (10 mg) proteinase K for 2 minutes, and then incubated at 55^{0}C for 30 minutes. After cooling to room temperature, the tissue/cell suspension was mixed with 2 mls of 5M NaCl and 2.5 mls of Oligo-dT and rotated at room temperature for 1-2 hours. After removing the supernatant by centrifugation at 1,000 rpm (J2-21 M/E; Beckman) for 5 minutes, the pellet was washed twice with 10 mls of RNA binding buffer and once of with 10ml of RNA wash buffer, and retrieved by centrifugation at 3,000 rpm (J2-21 M/E; Beckmen) for 3 minutes. The poly A$^+$ RNA was eluted from Oligo-dT cellulose by incubating with 2 mls of RNA elution buffer at 60^{0}C for 5 minutes. This elution step was repeated with 1.5 mls of RNA elution buffer. The eluants were combined and extracted once with 3ml of phenol/chloroform (1:1), and once with 3 mls of chloroform. The aqueous phase was separated by centrifugation at 5,000 rpm (J2-21 M/E; Beckman) for 5 minutes. The poly A$^+$ RNA was precipitated by incubating with 8 mls of 100% ethanol and 400 µl of 3M sodium acetate pH5.2) at –20^{0}C overnight, and pelleted by centrifugation at 10,000 rpm (J2-21 M/E; Beckman) for 40-60 minutes at 4^{0}C. The poly A$^+$ RNA pellet was washed with 70% v/v ethanol, air-died and resuspended in 200 µl of DEPC-treated 1 x TE. A 10 µl aliquot of the sample was diluted to 400 µl with TE for the determination of the poly A$^+$ RNA concentration and quality by UV spectrophotometer at OD_{260nm} and OD_{280nm} (Lambda Bio20; Perkin Elmer). Pure preparation of RNA has OD_{260nm}/ OD_{280nm} values of approximately 2.0. The remaining 190 µl of poly A$^+$ RNA was mixed with 550 µl of 100% v/v ethanol and 19 µl of 3 M sodium acetate (pH 5.2) and stored at –70^{0}C. The concentration of poly A$^+$ RNA was calculated by the following formula: Total amount of poly A$^+$ RNA (µg) in 760ul = OD_{260nm} x 304.

2.4-3 RNA Gel Electrophoresis

Total RNA (20 µg) or poly A$^+$ RNA (3 µg) was denatured in 10 µl of RNA loading buffer at 65^{0}C for 5 minutes, followed by fractionation on a formaldehyde gel containing 1% w/v agarose gel, 1 x MOPS, 0.63% v/v Formaldehyde, 1 µg of ethidium bromide, in 1 x MOPS electrophoresis buffer. The RNA gel was electrophoresed for 16 hours at 25 V or 5 hours at 75 V. The RNA was transferred to Hybond-C Extra nitrocellulose membrane (Amersham Pharmacia) using 20 x SSC as transferring buffer,

according to the manufacturer's instructions, and then fixed onto the membrane by baking at 80^0C in vacuum for 2 hours.

2.4-4 Northern Hybridization

The RNA-bound filter was prehybridized in 10 mls of a solution containing 50% v/v deionized formamide, 5 x SSC, 1 x Denhardt's, 300 µg/ml denatured herring sperm DNA, at 42^0C for 2 hours, and then hybridized with denatured (α-^{32}P) dCTP-labelled DNA probe in 10 mls of a solution containing 50% deionised formamide, 1% w/v SDS, 1M NaCl, 10% w/v dextran sulphate, 100 µg/ml denatured herring sperm DNA, at 42^0C overnight. The membrane was washed twice with 2 x SSC/0.1% w/v SDS, and twice with 0.2 x SSC/0.1% w/v SDS for 30 minutes each at 65^0C. The membrane was exposed to a Fuji phosphorimage screen overnight and visualized using a phosphorimage analyzer (FLA-2000, FUJIFILM).

2.4-5 Reverse Transcriptase PCR (RT-PCR)

For reverse transcription of total RNA and poly A^+ RNA, AMV reverse transcriptase was used (Promega-protocol as supplied). Nine µl (1 µg) of total RNA was directly used from the stock which was stored at -70^0C. Half µg poly A^+ RNA was precipitated by mixing with 1 µl tRNA (1 µg) and centrifuged at 10,000 rpm at 4^0C for 30 minutes, followed by an ice-cold 70% v/v ethanol washing step and centrifuged again at 10,000 rpm at 4^0C for 20 minutes. The poly A^+ RNA pellet was air-dried and resuspended in 9 µl DEPC-treated MQH_2O. The RNA solution was denatured at 70^0C for 10 minutes and cooled down on ice for 5 minutes. The denatured RNA was mixed with 5 mM $MgCl_2$, 1 x reverse transcription buffer, 1 mM dNTP's, 0.5 U rRNasin ribonuclease inhibitor (Promega), and 20pmol oligo-nucleotide (Oligo-dT) primer in a total volume of 20 µl. The reaction was incubated at 42^0C for 15-30 minutes. To stop the reaction, the reaction mixture was incubated at 99^0C for 5 minutes and cooled on ice. The mixture was diluted by adding 80 µl DEPC-treated MQH_2O. Two µl of the reverse transcription reaction was used in the polymerase chain reaction or LightCycler PCR.

2.4-6 Real Time LightCyler

The mRNA expression level of downstream target genes was analyzed by quantitative RT-PCR. The first strand cDNA was synthesized by Reverse Transcriptase kit (Promega) for each cell line as described by the manufacturer. A total reaction volume of 20 μl containing 2 μl of first strand cDNA, 3 mM $MgCl_2$, 10 pmole each primer, LightCycler RT-PCR Reaction Mix SYBR Green I (1x) and LightCycler RT-PCR Enzyme Mix (Roche) was used to assess the amount of each gene using the LightCycler (Roche). The denaturation and amplification conditions were 95^{0}C for 30 seconds followed by up to 40 cycles of PCR (10 seconds 95^{0}C, 10 seconds 55^{0}C and 15 seconds 72^{0}C). After amplification a melting curve was obtained by heating at 0.1^{0}C/second to 95^{0}C with fluorescence data collection at 0.1^{0}C intervals. The following primers were used for PCR:

EWS/FLI-1:	EWS-7S	5'-CAGCTATGGACAACAGAGTAG-3',
	Fli-18A	5'-GGATCTGATAAGGATCTGGC -3';
KF or *mKF*:		
	KRAB-SB	5'-GTGGACTTCACCAGGGAGGAG-3';
c-myc:	c-myc-S	5'-CTCAACGACAGCAGCTCGCC-3';
	c-myc-A	5'-GGAGACGTGGCACCTCTTGAG-3';
GAPDH:	GAPDH-S	5'-CTGCCACCCAGAAGACTGTGG-3',
	GAPDH-A	5'-GTCATACCAGGAAATGAG-3'.

These primer pairs result in PCR products of 281bp (*EWS/FLI-1*), 480 bp (*KF* or *mKF*), 312 bp (*c-myc*) and 460 bp (*GAPDH*).

Quantitative analysis of the LightCycler data was performed employing LightCycler analysis software (Roche). To compensate for variability in RNA amount and for exclusion of general transcriptional effects, the initial template concentrations of *EWS/FLI-1* fusion gene, *KF* or *mKF* fusion genes and c-myc gene were normalized to those of *GAPDH* control gene. Results are reported as specific-gene/*GAPDH* ratio value. Multiple reactions of real time semi-quantitative RT-PCR applied to two different total RNA pools of each cell line constituted the result of each gene mRNA expression levels.

2.5 Protein Manipulations

2.5-1 Protein Extraction

Cell cultures in log phase of growth were washed twice by PBS. After PBS buffer was removed, an appropriate amount of 1 x protein sample buffer was added. The cell lysate was scraped by a cell scraper (Nunc) and collected in a screw-capped tube. The lysate was then boiled in water for 5 minutes. The bulky particles were removed by centrifugation at 13,000 rpm for 5 minutes using a bench-top centrifuge.

2.5-2 SDS-polyacrylamide Protein Gel Electrophoresis (SDS-PAGE)

Thirty μl of cell lysate was loaded into each well (15 wells in total per gel) of an 8% SDS-polyacrylamide gel. The electrophoresis was carried out in a protein gel tank with 1 x SDS protein running buffer (model: Mighty Small II SE 250; Hoefer Scientific Instruments). The voltage was set at 50 V for ~30 minutes until the sample dye moved to the boundary of the stacking gel and the separating gel, and then changed to 100 V for 2 hours. BENCHMARK™ Prestained Protein Ladder (GIBCO-BRL) was used as a protein marker.

2.5-3 Protein Transfer

The SDS-polyacrylamide gel was washed in transfer buffer (1 x SDS and 20% methanol) before putting the gel into a transfer gel tank. The protein was transferred to Hybond-C Extra membrane (Amersham) by a semi-dry Western Gel Transfer Apparatus (Model: TE77; Pharmacia Biotech). All other procedures followed the manufacturer's instructions on the gel tank. The current 250 mA until 20 V was reached. The whole transferring process took 2 hours.

2.5-4 Western Blotting

After transfer, the filter was incubated in blocking buffer for 30 minutes at room temperature on a shaker. The primary antibodies (such as Fli-1 (C), c-myc, β-tub etc)

were diluted 1:1000 for overnight incubation at 4^0C in a sealed plastic bag. Excess antibody was washed off in 3 times SDS/0.01% TWEEN-20, 10 minutes each antibody (such as anti-rabbit, anti-goat and anti-mouse Abs) was diluted to appropriate dilution by 10 mls blocking buffer with the filter incubated at room temperature for 1-2 hours. The filter was washed 3 times in SDS/0.01% TWEEN-20 with 10 minutes each, and followed by 3 times of PBS with 1min each time to remove TWEEN-20 substance. The signal detection was carried out by submerging the filter in SuperSignal Chemiluminescent Substrate (PIERCE) for 15 minutes and exposed on a Kodak X-AR film with intensifying screens for 15 seconds to 5 minutes.

2.6 Mammalian Cells Manipulations

2.6-1 Cell Cultures

All cell lines were maintained at 37^0C and 5% CO_2 in Delbuccos Modified Eagles Medium (DMEM) (GIBCO-BRL) supplemented with 10% Fetal Calf Serum (FCS) (PA Biologicals Pty Ltd.). The media were changed every 2-3 days and supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml). The cell lines were split twice a week using a 1-minute treatment with 0.25% trypsin/EDTA (Sigma) to dislodge the adherent cells. Cell lines were maintained at subconfluent density on 100 mm diameter culture dishes (NUNCLON). Cell growth curves were determined as follows: cells were seeded into 3cm plates (2ml) at 5000 cells/ml for NIH3T3 cells and 1000 cells/ml for SK-N-MC with triplicate plates for each cell line at each time point. Four 48 hours time points were assessed for cell growth by counting using a hematocytometer. For the soft agar growth assay, cells were suspended in 0.2% agarose/DMEM/10%FCS and plated on a base of 1% agarose/DMEM/10%FCS. After 2 weeks 37^0C incubation, colonies with >20 cells were counted.

2.6-2 Freezing and Thawing Cell Lines

Cell cultures in log phase of growth were harvested using trypsin/EDTA as in Section 2.6-1. The dispersed cells were centrifuged at 2,000 rpm for 2 minutes and resuspended

in PBS. The washing step was repeated and the cells centrifuged as before. After removing the supernatant, the cells were resuspended in 900 µl FCS/100ul DMSO solution and transferred and sealed into a cyro-tube (Nunc). The tube was incubated overnight at -70^0C in a special foam container and transferred into a liquid nitrogen container (Thermolyne) for long-term storage. To thaw the frozen cell lines, the frozen tube was incubated at 37^0C for approximately 1min and the cells washed in 20ml normal media. After centrifugation, the cell pellet was resuspended then transferred to a tissue culture dish with the growth media and incubated at 37^0C in 5% CO_2.

2.6-3 Electroporation of NIH3T3 Cell Lines

Electroporation of 1 µg DNA into 10^5 NIH3T3 cells (resuspended in 0.5 mls RPMI with 10 mM Hepes) was carried out in 0.1 mls culture medium at 650 mF and 200 V in a 0.4 µl cuvette with the BIO-RAD Gene Pulser and Pulse Controller transfection apparatus. The transfected cells were allowed to recover for 24 hours in DMEM and 10%FCS before being placed into selective media. Selection of neomycin expressing transfectants was carried out in DMEM/10% FCS supplemented with G418 (400 µg/ml) for 14 days. Puromycin expressing transfectants were selected in DMEM/10% FCS supplemented with puromycin (5 µg/ml) for 7 days. Separate pools of neomycin or puromycin expressing cells were dispersed with trypsin/EDTA and used tc generate clonal cell lines by limiting dilution.

2.7 Mouse Tumour Formation Assay

The tumourigenic potential of each cell line was evaluated by injection of cell suspensions into 2-3 weeks old *BALB/c nu/nu* female mice. For each clone tested, five mice received subcutaneous injection of 2 x 10^6 cells (EWS/FLI-1 transformed NIH3T3 cell lines) or 5 x 10^6 cells (SK-N-MC cell lines) in a volume of 0.2 mls of PBS into both dorsal midline sides. The mice were housed in microisolator cages, given food and water ad libitum, and handled in a sterile laminar-flow hood. Tumour sizes were measured every 3 days using Vernier calipers along two perpendicular axes. The

volume of tumour sizes was calculated by using the following formula: (mean diameter)^3 x $\pi/6$.

2.8 Reporter Gene Transactivation Assay

2.8-1 Transient Transfection Experiments

Both EWS/FLI-1 transformed NIH3T3 and SK-N-MC cell lines were transiently transfected by the liposomal transfection method using Lipofectamine™ 2000 Reagent (GIBCO-BRL). Cells were trypsinised, counted and plated into 24-well plates (Falcon) at a density of 1-3 x 10^5 cells per well. On the day of transfection, cells were 90-95% confluent. Three hours prior to transfection, the culture medium was removed from the cells and replaced with fresh medium but lacking penicillin/streptomycin. A total of 1 μg of plasmid DNA (see Section 4.2-10) was diluted in 50 μl of the cell line's preferred medium, but in the absence of serum and penicillin/streptomycin. Two μl of Lipofectamine™ 2000 Reagent was dissolved for 5 minutes in 50 μl of the cell line's preferred medium, without serum and penicillin/streptomycin. The DNA solution and Lipofectamine™ 2000 Reagent were mixed and incubated for 30 minutes to allow the DNA to permeate the liposomes. One hundred μl of the suspension was added drop-by-drop to each well and mixed by gentle agitation. The cells were then incubated at 37^{0}C and 5% CO_2 for 36 hours before being assayed. Fresh growth medium was added 6 hours post-transfection.

2.8-2 Luciferase Reporter Gene Assay

After the growth medium was removed, the cells were washed twice in PBS supplemented with 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. One hundred and twenty five μl of this buffer was left in each well to provide the necessary ionic cofactors for the 125 μl of Luciferase Substrate Reagent™ (Boehringer Mannheim) subsequently added. Cells were resuspended to aid thorough lysis and 200 μl of this 250 μl mixture was transferred to an opaque 96-well microtitre plate (Falcon). This reporter gene system

facilitates the transcriptional activity of a promoter to be measured from the amount of light emitted by transfected cells. This is proportional to the amount of luciferase transcribed, when corrected for transfection efficiency. This ATP-dependent oxidative decarboxylation of luciferin by firefly luciferase emits light at a 562 nm wavelength and was quantified in a LumiCount^{TM} Luminometer (Packard-Becker) at a gain level of 1.0 and a read length of 7.0 seconds.

2.8-3 β-Galactosidase Assay

Concurrently, the remaining 50 µl of the above mixture (see Section 2.8-2) was reassigned to a transparent 96-well ELISA plate (Falcon). Fifty µl of 2 x β-gal assay buffer was added and incubated at 37^{0}C until a yellow colour developed, representing cleavage of the substrate O-nitrophenyl-β-D-galactopyranoside. Intensity was measured at 415 nm in a BIO-RAD Model 3550 Microplate Reader.

2.8-4 The Relative Luciferase Activity

The relative luciferase activity was computed by dividing the luciferase activity (arbitrary light units) by the β-galactosidase activity (arbitrary light units) for each individual transfections, before averaging each triplicate experiment. A percentage induction was then calculated by dividing the relative luciferase activity of each transfection reaction by the relative activity of the MMP3-luc or mutant MMP3-luc reporter constructs co-transfectants with the pSV-β-galactosidase control vector transfectants.

Chapter 3

Transformation of NIH3T3 Fibroblasts by Murine *EWS/Fli-1* Fusion Gene

3.1 Introduction

Although the survival rate of patients with ESFT have improved over the past decades, difficulties with accurate prognosis, side effects of treatments, minimal residual disease, low survival after relapse and lack of innovative treatments have hindered the improvement of clinical outcome. Molecular analysis suggests that the EWS/FLI-1 fusion protein has an important role in ESFT tumourigenesis, although the precise aetiology and mechanisms of this fusion protein in tumour development are still unclear. The human EWS/FLI-1 fusion protein has been shown to be an oncoprotein in transforming this fibroblast cells by forming colonies in semi-solid agar (May *et al*, 1993a) and tumours in severe combined immunodeficient (SCID) mice (May *et al*, 1997a). However, these data have only provided *in vitro* evidence of EWS/FLI-1 as a transcription factor or oncogene. An ideal approach for understanding the function of ESFT would be the analysis of a mouse model for PNET to provide evidence of the mechanisms of transformation *in vivo* and ultimately reveal new therapeutic strategies. Unfortunately, no such model exists and there is no evidence that a similar fusion protein in another species would cause oncogenesis. Thus, to generate an appropriate mouse model for ES/PNET, murine EWS/Fli-1 needs to display similar biochemical characteristics as human EWS/FLI-1.

The mouse and human *EWS* and *Fli-1* genes share 98% and 97% homology respectively and show similar expression patterns (Prasad *et al*, 1992; Plougastel *et al*, 1994). In this study we have artificially fused the mouse *EWS* and *Fli-1* genes to mimic the human type I *EWS/FLI-1* fusion gene. The resultant murine *EWS/Fli-1* fusion gene shares 98%

homology to the human fusion gene. The tumourigenicity of mouse and human EWS/FLI-1 had been compared by soft agar assay, low serum medium and tumour formation in *BALB/c nu/nu* mice.

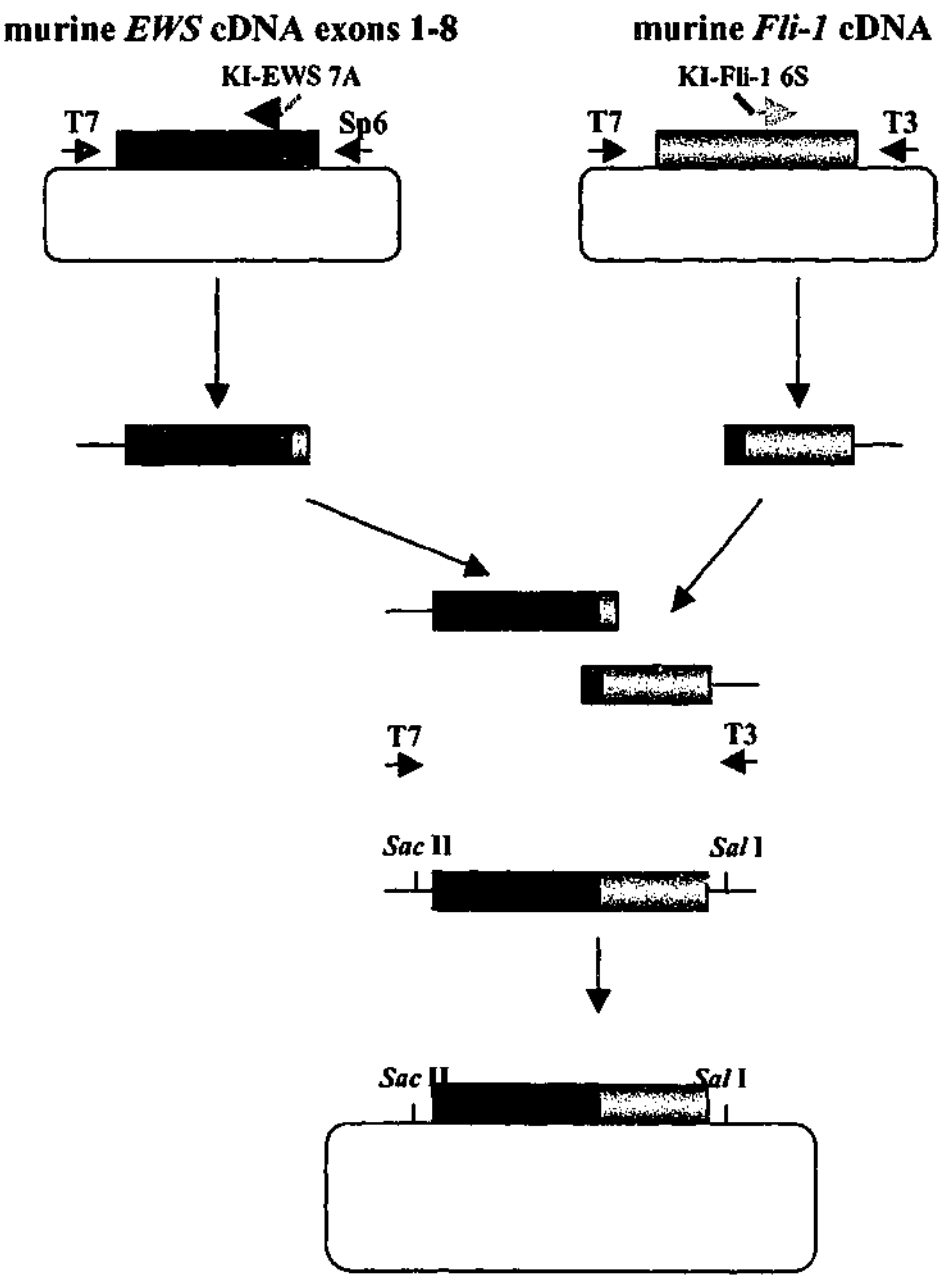
It is also important to demonstrate that murine EWS/Fli-1 transformation is associated with activation of a similar range of genes as shown for human EWS/FLI-1 *in vitro*. Human EWS/FLI-1 has been shown to display similar biochemical characteristics to FLI-1 such as nuclear localisation and binding DNA in a site-specific manner (May *et al*, 1993b, Bailly *et al*, 1994; Mao *et al*, 1994). However, the EWS transactivation domain in EWS/FLI-1, which transactivates reporter genes more efficiently than FLI-1, potentially dysregulates a set of FLI-1 downstream targets. These genes result in the transformation observed in ESFT (Lessnick *et al*, 1995). By RNA Differentiation Assay (RDA), many genes have been shown to be upregulated by human EWS/FLI-1 (Bailly *et al*, 1994; Braun *et al*, 1995; Thompson *et al*, 1996; May *et al*, 1997; Arvand *et al*, 1998), some of which are implicated in control of cell growth, differentiation, and oncogenesis and include genes such as *c-myc*, *stromelysin-1*, *Manic Fringe* and *EAT-2*. Thus we have determined the expression of these genes at the mRNA and protein levels in cells transformed by either the human or murine EWS/FLI-1 fusion proteins.

3.2 Results

3.2-1 Generation of a Murine *EWS/Fli-1* Type 1 Fusion Gene

To generate partial mouse *EWS* cDNA, a RT-PCR strategy was used to isolate the appropriate sequence from mouse embryonic stem cell (C57B1/6J) mRNA. Briefly, the first strand cDNA was synthesised using oligo-dT (Boehringer Mannheim) and AMV reverse transcriptase kit (Promega). The 5' primer, MEWS-1S (-11 from initiation codon) and the 3' primer, MEWS-8A, (+937 at exon 8) (see Appendix 4) were used to amplify a 1.0 kb *EWS* cDNA fragment on the first strand cDNA library. *Elongase* (Life Technology), a mixture of *Taq* and *Pyrococcus* species GB-D thermostable DNA polymerase, was used to minimize the introduction of mutations. After amplification, an adenosine (dATP) tail was added to the amplified fragment by *Taq* polymerase to allow more efficient cloning into the pGEM-T vector. Appropriate clones were identified by restriction enzyme digestion and sequenced by T7 and SP6 primers at pGEM-T vector and were proved to be identical to the murine *EWS* cDNA sequence on the GeneBank database (Appendix 5). The 1.73 kb murine *Fli-1* cDNA (provided by Drs. A Hart and A. Bernstein) was subcloned into the *Eco*RI site of pBluescript II SK+ vector. The cDNA was shown to be correct by sequencing with T7 and T3 primers (Appendix 6).

The type I *EWS/FLI-1* translocation fuses exon 7 of *EWS* to exon 6 of *FLI-1* gene and is the most predominant translocation in ESFT (Zucman *et al*, 1993). Thus a murine *EWS/Fli-1* fusion gene was designed to mimic the human type I *EWS/FLI-1* fusion sequence. This murine fusion was generated by using an overlap extension PCR technique (Fig. 3.1). The first step used T7 and KI-EWS 7A primers (Fig. 3.1) (Appendix 4) to PCR amplify the murine *EWS* partial cDNA (exons 1 to 7) from the vector described above. The fragment generated contains 864 bp with a tail at the exon 7 and contains 20 bp of murine *Fli-1* exon 6 . The second step used T3 and KI-Fli-1 6S primers to amplify a murine *Fli-1* cDNA fragment (exons 6 to 9) for the cDNA clone described above. This fragment contained 835 bp with 20 bp attached to exon 6 (murine *EWS* exon 7). To generate the murine *EWS/Fli-1* fusion gene, both PCR products were combined and PCR amplified using T7 and T3. The murine *EWS/Fli-1* fusion gene (1.6 kb) fragment was purified by agarose gel electrophoresis and Gene Clean (see Section



	EWS region	*Fli-1* region
KI-EWS 7A	5'-GCTACGGGCAGCAGAACCCTTCTTATGACTCTGTC-3'	

	Fli-1 region	*EWS* region
KI-Fli-1 6S	5'-AGTCATAAGAAGGGTTCTGCTGCCCGTAGCTGCTG-3'	

Fig. 3.1 Schematic diagram showing the cloning of murine *EWS/Fli-1* cDNA by overlapping PCR strategy. The 2nd PCR product was subcloned into pBluescript K+ vector. The murine *EWS/Fli-1* fusion sequence was verified by T7 and T3 primers in pBluescript KS + vector.

2.2-3). After digestion with *Sac*II and *Sal*I, this fragment was subcloned into the pBluescript II SK+ vector. The correct sequence of this murine EWS/Fli-1 fusion gene was confirmed by sequencing using T7 and T3 primers (Appendix 7).

3.2-2 Expression Constructs for Murine and Human EWS/FLI-1 Fusion Proteins

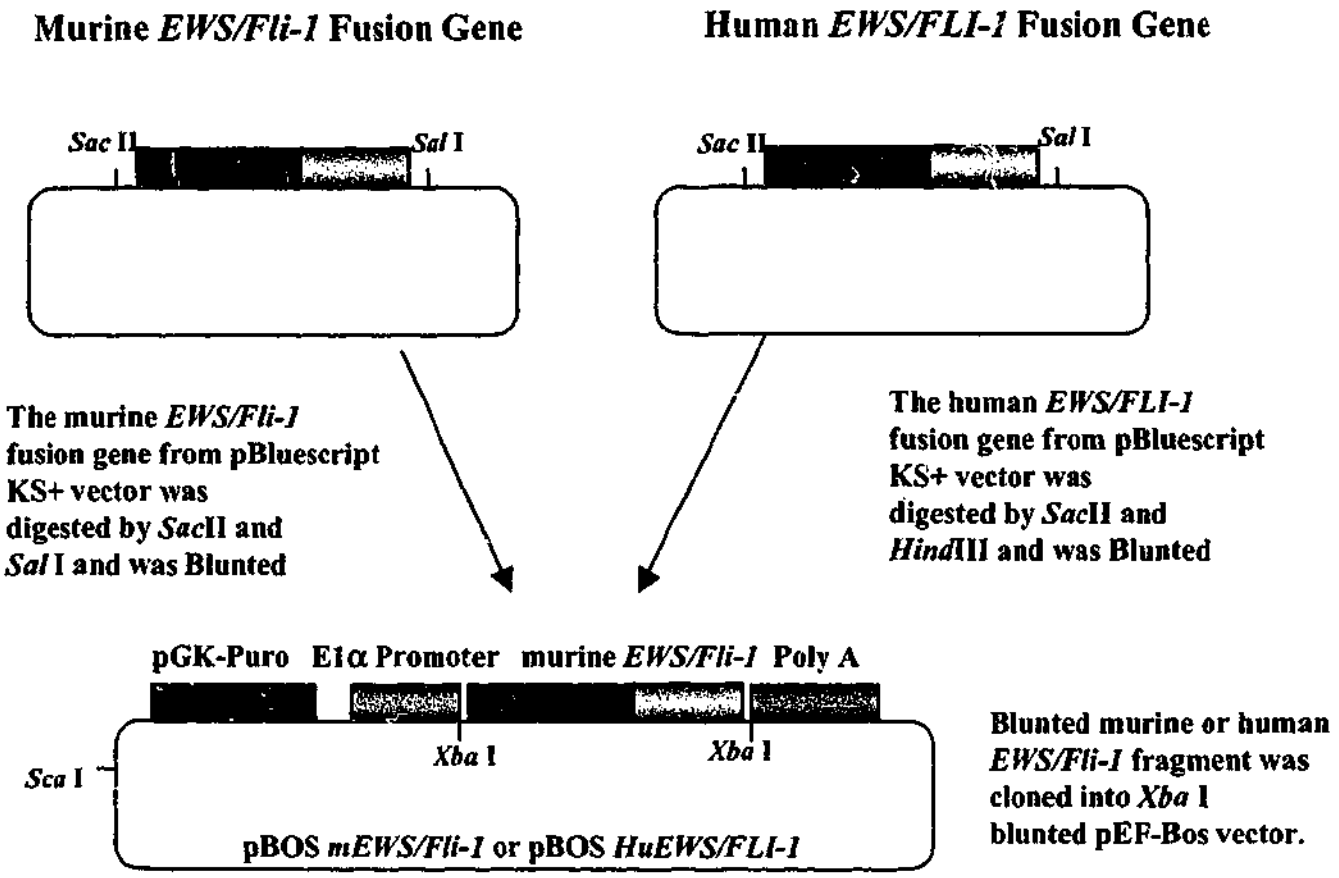
To stably express murine *EWS/Fli-1* or human *EWS/FLI-1* (Appendix 9) fusion gene in fibroblast cells, a promoter with high expression ability in eukaryotic cells was required similar to the *EWS* promoter. Thus the human elongation 1α (human E1α) promoter and human cytomegalovirus (CMV) minimal promoter were used. These two promoters have been reported to be strong promoters for gene expression in eukaryotic cell lines (Pasleau *et al*, 1985; Mizushima *et al*, 1990; Malherbe *et al*, 1992). In addition, the *puromycin* gene was cloned into vectors containing these promoters for antibiotics selection of cells with these constructs integrated (Fig. 3.2).

The murine *EWS/Fli-1* fusion gene in pBluescript SK+ generated above was excised by using the restriction enzymes *Sac*II and *Sal*I, blunted by *Klenow* enzyme and subcloned into *Xba*I filled-in site of the pEF-BOS vector (Mizushima *et al*, 1990) (Fig. 3.2). Similarly the human type 1 *EWS/FLI-1* fusion cDNA in pBluescript II SK+ was digested by *Sac*II and *Hind*III, blunted using *Klenow* (Promega) and subcloned into *Xba*I filled-in site of the pEF-BOS vector (Fig. 3.2). The orientation of both fusion genes was confirmed by restriction enzyme analysis and sequencing using T7 and T3 primers. Similar constructs have also been generated using the cytomegalovirus (CMV) minimal promoter by subcloning into the *Kpn*I filled-in site of pBluescript KS + vector containing CMV promoter (Fig.3.2).

3.2-3 Generation and Analysis of Cell Lines Transfected with the Murine and Human *EWS/FLI-1* Fusion Genes

The murine and human *EWS/FLI-1* expression constructs were each linearized by *Sca*I and transfected into NIH3T3 cells by electroporation. After 1-week selection using 5

A) Bos (Human E1α) Promoter Expression Constructs



B) Human CMV Promoter Expression Construct

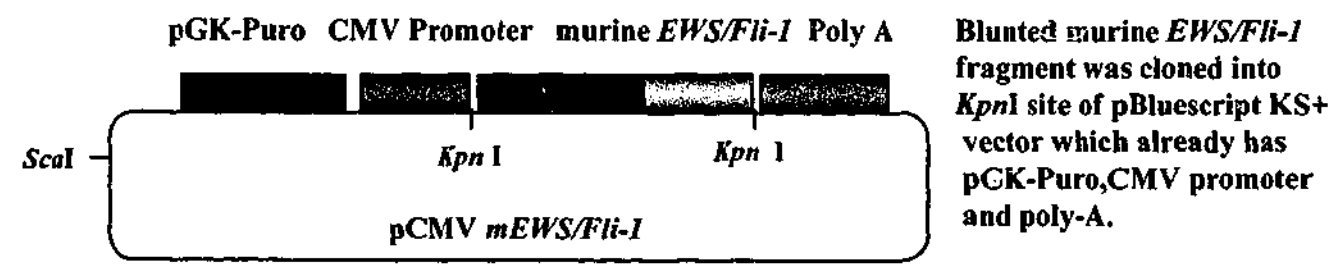


Fig.3.2 Schematic diagram showing the cloning of murine and human *EWS/Fli-1* fusion genes into expression vector. (A) Murine or human *EWS/Fli-1* fusion gene fragment were excised and blunted from pBuescript KS+ vector and subcloned into pEF-Bos vector. (B)Murine *EWS/Fli-1* fusion gene was cut and subcloned into CMV expression vector. All constructs were sequenced to verify correct insertion and orientation.

μg/ml puromycin, resistant clones were isolated by carefully picking under a stereomicroscope. Each clone was then expanded and analysed for expression by Western blot and RT-PCR analysis. The promoter vector with *puromycin* resistant gene was similarly transfected into NIH3T3 cells to generate control cell lines.

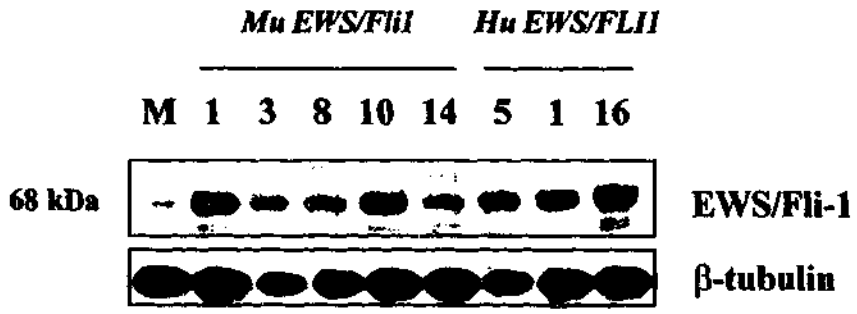
To examine the expression of murine and human EWS/FLI-1 fusion proteins in puromycin resistant clones, a polyclonal antibody Fli-1 (C-19) which recognizes the C-terminal region of Fli-1 protein was used in Western analysis (Santa Cruz Biotechnology, Inc. CA. Cat. # sc-356). These results identified that 6 out of 10 clones from the pBos *mEWS/Fli-1* transfection and 8 out of 10 clones from the pCMV mEWS/Fli-1 transfection expressed the expected 68 kDa band (Fig. 3.3). Similar to murine EWS/Fli-1 positive clones, 9 out of 10 pBos *HuEWS/FLI-1* transfected clones were shown to have the 68 kDa band (Fig.3.3). A faint band of similar size was also observed in control cells and is due to cross-reactivity of the Fli-1 polyclonal antibody used (Fig.3.3). By comparing with the expression level of β-tubulin protein as a loading control, there were variable expression levels between each of the murine and human *EWS/FLI-1* expressing clones. For some selected murine *EWS/Fli-1* expressing clones, mEF#1 (Bos) had the highest expression compared with others such as mEF#8, mEF#10 and mEF#14 (CMV) (Fig.3.3). For some selected human *EWS/FLI-1* expressing clones, HuEF#1 and HuEF16 had a higher expression level than HuEF#4, HuEF#5 and HuEF#7 (Fig.3.3).

Furthermore, all Western positive clones of murine and human EWS/FLI-1 were also positive by RT-PCR analysis. A 5' primer, MEWS-6S (at exon 6 of EWS), and a 3' primer, MFli-1 8A (at exon 8 of Fli-1) (Appendix D), were used to amplify a 400 bp fragment. RT-PCR of two representative clones from murine and human EWS/FLI-1 transfections are shown in Fig. 3.3.

3.2-4 Growth Rate of Murine and Human *EWS/FLI-1* Transfectants in Normal and Low Serum Medium

Transformed cells, unlike normal cells, are able to proliferate in low serum culture medium as they become independent of growth factors present in serum. Two

A) Western Analysis



B) Semi-quantitative RT-PCR

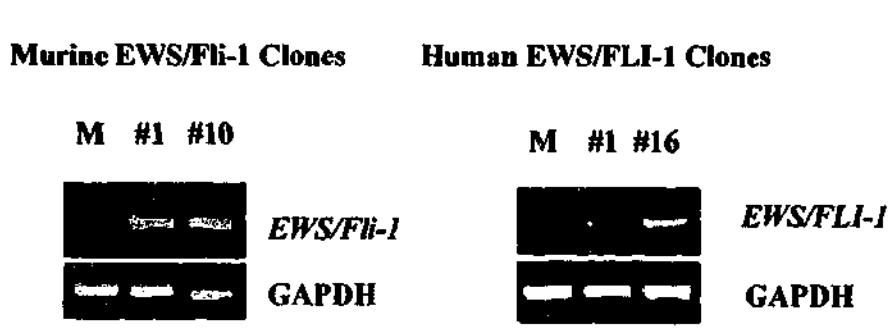


Fig.3.3 The expression of murine or human EWS/FLI-1 fusion protein/mRNA by (A) Western blot and (B) Semi-quantitative PCR.

representative human EWS/FLI-1 clones HuEF#1 and HuEF#16, three representative murine EWS/Fli-1 clones mEF#1, mEF#10 and mEF#14, and normal control cells were seeded in triplicate in 3 cm wells in Dulbecco's modified Eagle's medium (DMEM; GIBCO) and supplemented with either 10% FCS (normal) or 1% FCS (low serum) (5 x 10^3 cells of each cells). The cell proliferation rates were measured by direct cell counting of single-cell suspensions which stained with Trypan blue for 12 days at 2 day-intervals. Results are shown as means $\pm$ S.D. from 3 independent experiments.

All clones including normal control cells proliferated at similar rates in normal media (data not shown). However, only murine or human *EWS/FLI-1* expressing clones proliferated in a rapid rate in low serum medium (1% FCS) from day 1 to day 12 (Fig.3.4). Interestingly, clones with a high expression of EWS/Fli-1/EWS/FLI-1 clones had a higher proliferation rate compared with others. For example, HuEF#1, HuEF#16 and mEF#1, which had been shown to have higher EWS/FLI-1 protein levels (see Section 3.2-3) proliferated faster than mEF#10 and mEF#14 (Fig.3.4). The normal control cells were not able to grow in low serum medium and all subsequently died (Fig. 3.4). These data demonstrate that both murine or human *EWS/FLI-1* fusion proteins altered NIH3T3 cells to be less dependent of growth factors present in fetal calf serum. Furthermore, the relative amount of murine or human EWS/FLI-1 proteins correlated with an increased proliferation rate in low serum medium.

3.2-5 Growth in Soft Agar

Another characteristic of transformed cells *in vitro* is that they often become anchorage independent and are able to grow soft agar. This further investigate murine or human EWS/FLI-1 transformed NIH3T3 cells, 5 of murine EWS/Fli-1 clones and 3 of human EWS/FLI-1 clones were studied using a soft agar assay. For the soft agar assays, 5,000 cells of each clone was mixed with media containing 0.3% agarose gel and seeded in triplicate in 3 cm wells on top of a 1% agar media mixture (Promega). After 2 to 3 weeks, the cultures were scored for viable colonies. Three separate experiments were performed for each clone.

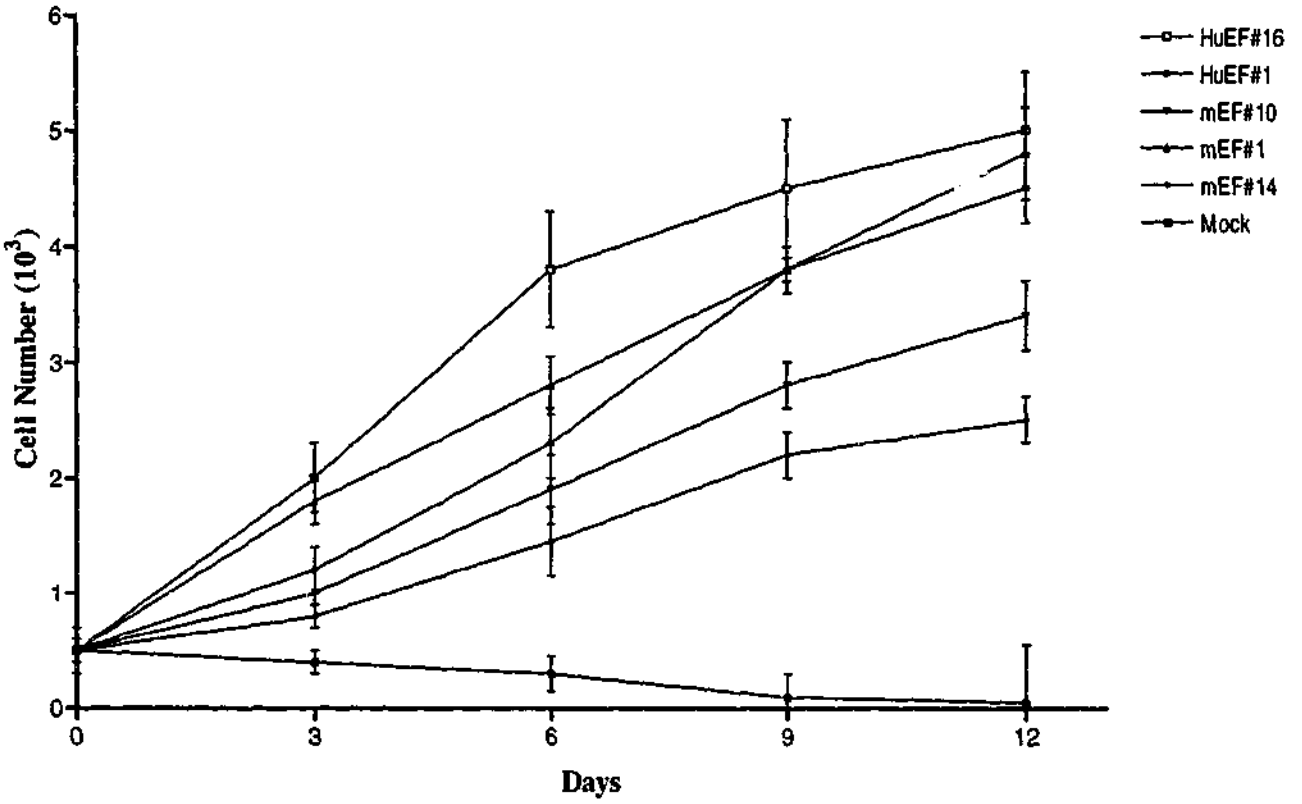


Fig.3.4 The proliferation rate of EWS/Fli-1 transformed cell lines in low serum medium. The cell number was counted by using Hematocytometer for 3 independent cultures and cells were cultured in three 3-cm culture plates for each time.

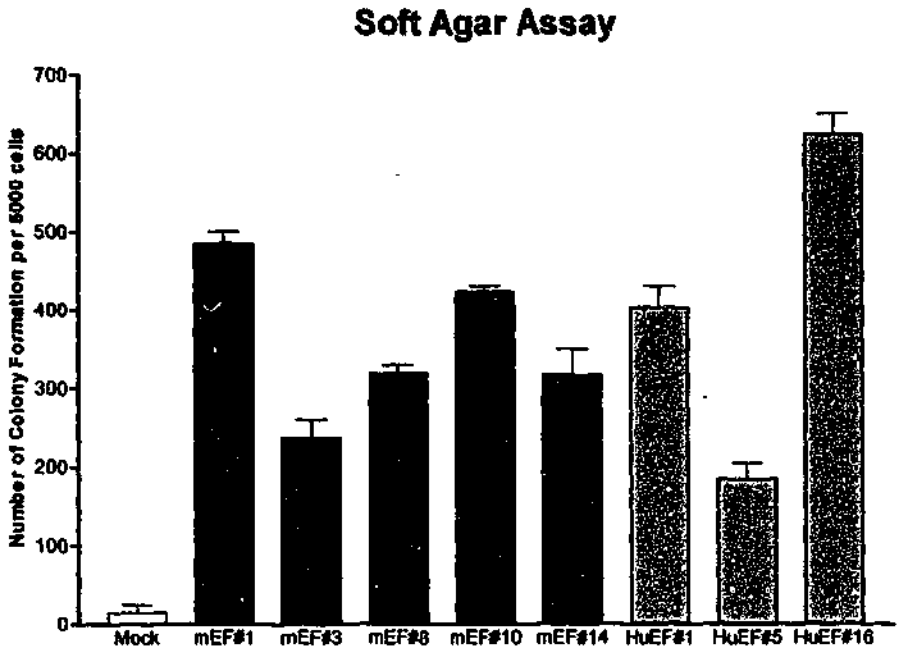
All 8 human EWS/FLI-1 and murine EWS/Fli-1 expressing clones tested grew efficiently in soft agar, showing they were capable of growing in an anchorage-independent manner (Fig. 3.5). However, the normal control cells could not form colonies in the soft agar (Fig.3.5). The morphology of the colonies formed from murine and human EWS/FLI-1 expressing clones was similar (Fig.3.5), but the level of expression of the fusion protein appeared to correspond to the efficiency of colony formation (Fig.3.5). This was consistent with the results found in the low serum assay (see Section 3.2-4). For example, mEF#1 had the highest murine EWS/Fli-1 protein level and the number of colonies in soft agar was higher compared with other murine EWS/Fli-1 expressing clones (Fig.3.5). For human *EWS/FLI-1* transfected clones, HuEF#1 and HuEF#16 expressed relatively higher levels of fusion protein and also formed higher number of colonies in soft agar (Fig.3.5). Taken together, these data provide further *in vitro* evidence for the transformation of NIH3T3 cells by murine or human EWS/FLI-1.

3.2-6 Potential to form Tumours in Nude Mice

In order to study the ability of both murine and human *EWS/FLI-1* transfected cells to form tumours *in vivo*, each cell line was evaluated by injecting cell suspensions into age and sex matched *BALB/c nu/nu* mice. In this study, 4 murine and 2 human *EWS/FLI-1* transfected clones previously shown to express EWS/FLI-1 were examined. The *c-mos* transfected NIH3T3 cell line (kindly donated by Dr. A Seth) was used as positive control and normal control cell line (mock) was used as negative control. For each clone tested, one to 6 mice received subcutaneous injections of 2×10^6 cells in a volume of 0.2 ml of phosphate-buffered saline into both dorsal midline sides.

Four mice were injected with *c-mos* and mock cell lines while at least 5 mice for each murine and human EWS/FLI-1 expressing cell lines. All the mice injected with *c-mos* cells, murine or human EWS/FLI-1 transformed cells formed tumours which were observed but no tumours were found in those sites inoculated with control cells (Fig.3.6). Mice inoculated with clones expressing relative high levels of EWS/FLI-1 proteins such as mEF#1 and Hu#16, were observed to form palpable tumours 5-7 days after those inoculated with *c-mos* transformed cells which had palpable tumours from

A) Soft Agar Assay



B) Morphology of Colonies on Soft Agar

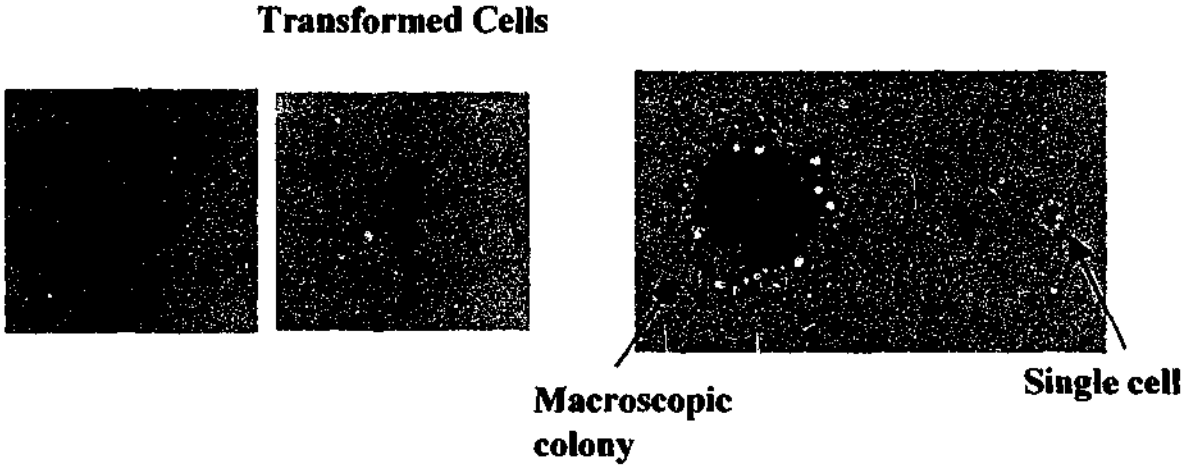


Fig.3.5 Soft Agar assay of murine and human *EWS/FLI-1* expressing cell lines. (A) The graphshown the number of colony formation in soft agar for each murine and human EWS/FLI-1 positive cell line compared with mock transfected cells. Five thousand cells were seeded in 0.3% agarose gel supplemented with 10%FCS/DMEM and cultured for 2 weeks until 20-cell of colony of >20 cells were observed. (B) Morphology of colonies on soft agar.

Nude Mice Assay

Cell Lines	Tumour Volume (mm^3)(week6)	No. of Injection
Mos	1200	8/8
mEF#1	760	20/20
mEF#8	480	10/10
mEF#10	680	10/10
mEF#14	280	10/10
HuEF#1	580	16/16
HuEF#16	890	20/20
mock	0	0/20

Fig.3.6 Nude mice assay. The photo shows the formation of tumours in *BALB/c nu/nu* mice. Cell lines including *mos* cells (positive control), $2x10^6$ cells in 200 µl PBS was subcutaneously injected into both flasks of *BALB/c nu/nu* mice. Tumours were observed in all mice injected with *mos* cell line, mouse and human EWS/FLI-1 expressing cell lines. Mos positive control cell line formed tumour since week 2 and had largest tumour size at week 6. The cell lines with high levels of mouse and human EWS/FLI-1 such as Bos mEF#1 and Bos HuEF#16 formed tumours since week 3 and had larger tumour size at week 6. Other *EWS/FLI-1* transfected cell lines had tumours initially observed from week 3.5 to 4 and had smaller tumour size at week 6. There was no development of palpable tumours for the mock transfected control cell line.

day 12 (data not shown). Clones with a lower expression of fusion protein (e.g. mEF#1, mEF#14 and HuEF#1) formed palpable tumours 2 weeks after the *c-mos* transformed cells (data not shown). Morphologically, the tumours formed by either murine or human *EWS/FLI-1* transfected cell lines were all well vascularized. To verify these tumours originated from *EWS/FLI-1* expressing cells, some tumour tissues were removed form different tumours and re-cultured them in complete medium. By Western and RT-PCR analysis, these tumour cells were shown to murine or human EWS/FLI-1 protein or mRNA respectively (data not shown). This shown that mouse and human *EWS/FLI-1* transfected cells are capable of forming tumours *in vivo* and the relative levels of EWS/Fli-1 expression in the transfected cell lines could affect tumourigenic potential.

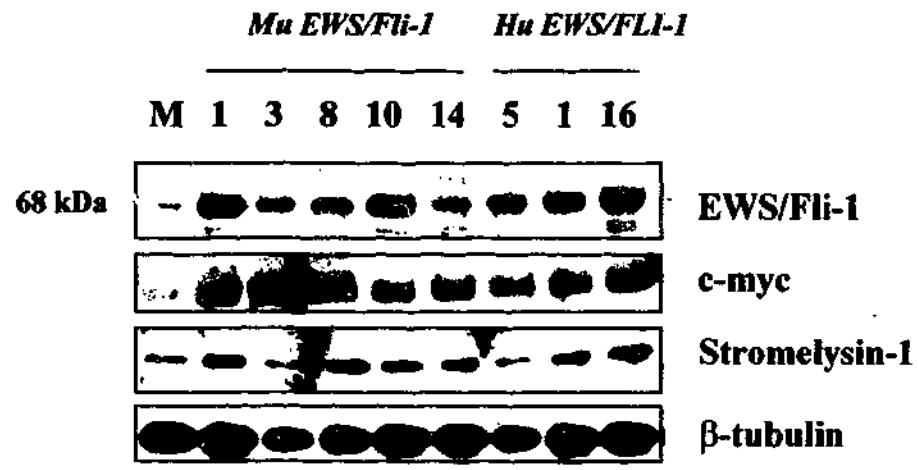
3.2-7 Up-regulation of Downstream Targets in Murine *EWS/Fli-1* Expressing Cell Lines

To demonstrate that murine EWS/Fli-1 transformed by activating a similar range of genes as that shown for human EWS/FLI-1, several genes shown to be activated in human EWS/FLI-1 expressing cells such as *c-myc*, *stromelysin-1* and *EAT-2* (Bailly *et al*, 1994; Braun *et al*, 1995; Thompson *et al*, 1996; May *et al*, 1997) were selected for analysis in this study by Western blot and/or semi-quantitative RT-PCR.

By Western blot analysis, all murine or human *EWS/FLI-1* expressing cell lines showed higher expression levels of c-myc and stromelysin-1 compared with normal cell controls (Fig.3.7). For the murine and human *EWS/FLI-1* transfected clones such as mEF#1, HuEF#1 and HuEF#16, the c-myc and stromelysin-1 protein levels were higher that the normal control and other clones (Fig.3.7). This data were consistent with the EWS/FLI-1 levels and increased proliferation rate of these clones.

To measure the mRNA expression levels of these genes downstream of EWS/FLI-1, specific primers for *c-myc* and *stromelysin-1* and *EAT-2* (Appendix D) were used in semi-quantitative PCR. Four representative clones of murine and human *EWS/FLI-1* expressing clones were analysed. The mRNA levels of *c-myc* and *stromelysin-1* in mEF#1, mEF#10, HuEF#1 and HuEF#16, which had been shown to express high levels of murine or human EWS/FLI-1 in Western blot were higher than the control cell line

A) Western Analysis



B) Semi-quantitative RT-PCR

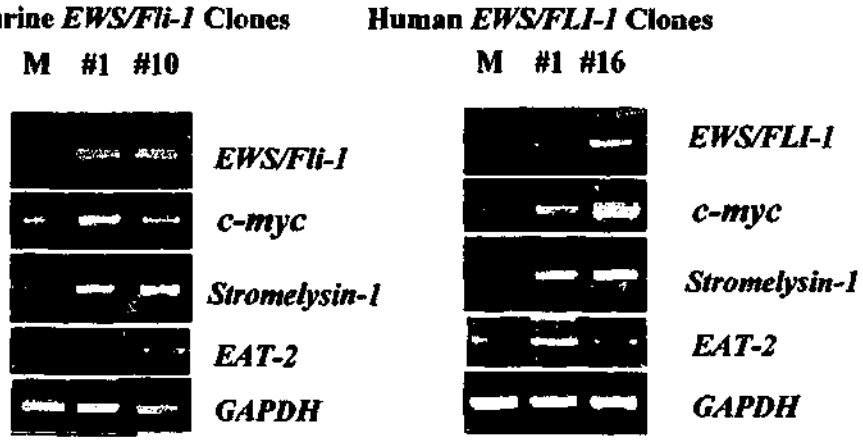


Fig.3.7 Western and Semi-quantitative RT-PCR showing expression of murine or human *EWS/FLI-1* and a selection of genes known to be activated in ES/PNET. (A) Western analysis on cell lysates of murine or human *EWS/FLI-1* expressing cell lines using Fli-1 (C), c-myc, Stromelysin-1 and β-tub polyclonal antibodies (B) semi-quantitative RT-PCR showing the expression of murine or human *EWS/FLI-1* transcripts, *stromelysin-1*, *c-myc* and *EAT-2*.

(Fig.3.7). These results were consistent with the protein levels observed on Western blot of these two genes. Another upregulated gene in PNET, *EAT-2*, was also measured by semi-quantitative RT-PCR (EAT-2 specific primers, see Appendix D). The mRNA level of this gene was also increased in mEF#1, mEF#10, HuEF#1 and HuEF#16 clones compared with the normal control cell line (Fig.3.7). Taken together, these data indicated both murine and human *EWS/FLI-1* fusion genes have the potential to transform in *in vitro* and *in vivo* experiments and this transformation is associated with the upregulation of the same genes in NIH3T3 cells which have been shown to be activated in ES/PNET.

3.3 Discussion

Although the precise aetiology of ESFT is still unclear, the human EWS/FLI-1 has been suggested to be a key role in the pathogenesis of these tumours (Zucman *et al*, 1993; Sorensen *et al*, 1994). Human EWS/FLI-1 has also been shown to efficiently transform NIH3T3 cells by forming colonies in soft agar and tumours in nude mice, suggesting that this aberrant protein acts as an onco-protein (May *et al*, 1993b). In this study, the murine EWS/Fli-1 which mimics to human EWS/FLI-1 was artificially generated and was the first time to showed to possess similar tumourigenic potential to human EWS/FLI-1. This included anchorage-independent growth in soft agar, proliferation rate in low serum media and tumour development in *BALB/c nu/nu* mice.

The human *EWS/FLI-1* fusion genes are heterogeneous with three different breakpoints within introns 3-9 of *FLI-1* and two breakpoints within introns 7-10 of *EWS* gene. This results in four alternate fusion proteins (Delattre *et al*, 1992; May *et al*, 1993). Among cases of ES/PNET with *EWS/FLI-1* translocations, the type I *EWS/FLI-1,* which fuses exon 7 of the *EWS* gene to exon 6 of the *FLI-1* gene, is the most predominate. This is observed in 60-70% of t(11;22) translocations (Ladanyi, 1995). The murine *EWS/Fli-1* fusion gene was thus mimicking human type I *EWS/Fli-1* fusion gene by overlapping PCR technique. Since the human *EWS/FLI-1* fusion gene expressed depends on the potent endogenous *EWS* promoter in ESFT cells, the murine EWS/Fli-1 fusion gene was also driven by the strong expression promoters, human E1α (Bos) or CMV promoters, in this study.

The murine EWS/Fli-1 fusion protein performed oncogenic ability similar to the human EWS/FLI-1 fusion in NIH3T3 cells, indicating they are onco-proteins. NIH3T3 mouse embryo fibroblasts are often used for transformation assays. Many genes such as *ras*, *c-myc*, *cdc 42* (involved in regulation of cell cycle) and *c-erg* have been identified as oncogenes by their ability to transform NIH3T3 cells *in vitro* and form tumours in nude mice *in vivo* (Blair *et al*, 1981; Hart *et al*, 1995; May *et al*, 1997b). In this study, all murine EWS/Fli-1 or human EWS/FLI-1 expressing cell lines proliferated faster in low serum medium and formed colonies in soft agar compared with normal control cell lines. This shown that both murine and human EWS/FLI-1 fusion onco-proteins can

induce the NIH3T3 cells to survive and grow in a medium with less growth factors and become anchorage-independent growth in semi-solid medium. Additionally, these cell lines except normal control cell lines also formed tumours in nude mice with a comparable frequency to that of NIH3T3 cells transfected with *c-Mos* proto-oncogene lined to the 5' long terminal repeat of the Moloney murine sarcoma virus (Blair *et al*, 1981). These data showed that both murine *EWS/Fli-1* and human *EWS/FLI-1* are onco-proteins with mitogenic and transforming potential when expressed in NIH3T3 cells.

The data in this study also shown that the relative amount of EWS/FLI-1 fusion proteins, in murine or human *EWS/FLI-1* expressing cell lines, give comparable tumourigenecity in NIH3T3 cells in terms of growth rate, colonies in soft agar and tumour formation in nude mice. This variable amount of fusion proteins was due to the different copy numbers of either murine or human *EWS/FLI-1* fusion genes in NIH3T3 cells which was shown by Western blot and semi-quantitative RT-PCR analysis. The higher expression of these fusion proteins, the higher oncogenic potential such as increasing proliferation rate in low serum, growth rates in soft agar and nude mice of EWS/FLI-1 transformed NIH3T3 cells. This result is correlated to the findings in ES/PNET cell lines; the SK-N-MC cell line has 23-folds of EWS/FLI-1 protein levels compared with SK-ES-1 cell line and thus SK-N-MC proliferate much faster than SK-ES-1 (Tanaka *et al*, 1997). Thus, these data further confirmed that the EWS/FLI-1 fusion proteins playing important roles in tumourigenicity in EWS/FLI-1 transformed NIH3T3 and ES/PNET cells as the relative amount of these fusion proteins can induce the variable oncogenic potential of ES/PNET cells.

To further confirm that the murine EWS/Fli-1 transforming NIH3T3 in the same mechanisms as the human EWS/FL-1 does, several downstream target genes which have been shown to be upregulated by human EWS/FLI-1 were investigated in this study. As described in Chapter 1, human EWS/FLI-1 induces transformation in NIH3T3 cells or ES/PNET by dysregulating the downstream targets of FLI-1. According to previous reports, these downstream targets involved *stromelysin-1*, *c-myc*, *Manic Fringe*, *mE2-C* and *EAT-2* have been shown to be up-regulated by human EWS/FLI-1 fusion proteins (Bailly *et al*, 1994; Braun *et al*, 1995; Thompson *et al*, 1996; May *et al*, 1997b). Due to their importance on cell cycle, cell proliferation and tumourigenic control (Bailly *et al*, 1994; Braun *et al*, 1995; Thompson *et al*, 1996), they were chosen

as oncogenic markers to be studied the difference between murine and human EWS/FLI-1. Under Western blot and semi-quantitative RT-PCR analysis, *stromelysin-1*, *c-myc* and *EAT-2* were shown increased expression levels in mRNA and protein in murine and human EWS/FLI-1 transformed cells compared with normal control cells. There was no difference between murine and human EWS/FLI-1 in upregulating these downstream targets, suggesting that both on murine and human EWS/FLI-1 transform NIH3T3 cells in the same oncogenic pathways. Furthermore, the high expression levels of EWS/FLI-1 showed higher efficiency in upregulating these downstream targets. This is because the more fusion proteins will involve in regulation of these downstream targets by prolonging the binding and transactivation time on promoters of downstream target genes. Ultimately, this increases transcription activities in production of more downstream oncogenes. The high expression levels of these downstream target genes correlate the transformation phenomena in the high EWS/FLI-1 expressing clones. This further demonstrates that the EWS/FLI-1 fusion protein is a single potent factor in transformation of NIH3T3 cells and ES/PNET.

Although the treatment strategies in ESFT have improved over the past decades, the survival rate is still poor and the aetiology of this tumour is unclear. Thus, more innovative treatments to improve clinical outcome are still necessary in this tumour. Besides, the investigation of the aetiology and molecular mechanisms in this tumour is the direction for the treatment of this tumour. There are lots of evidence showing human EWS/FLI-1 is the key role in tumourigenesis in ESFT (Delattre *et al*, 1992; Zucman *et al*, 1993; May *et al*, 1993a and b; Sorensen *et al*, 1994; Lessnick *et al*, 1995). However, all these data come from *in vitro* experiments which cannot completely explain the functions of EWS/FLI-1 in tumourigenesis of ESFT. A mouse model mimicking to human ESFT is necessary to provide a better *in vivo* data on this fusion protein. In this study, the data clearly show that murine EWS/Fli-1 could transform NIH3T3 cells similar to human EWS/FLI-1 in increasing proliferation rate, growth rates in colonies formation in soft agar and tumour formation in nude mice. The most important finding was that both murine and human EWS/FLI-1 can upregulating *c-myc*, *stromelysin-1* and *EAT-2* in fusion protein dependent manner, indicating that both fusions dysregulating the FLI-1 downstream target genes and recruit similar oncogenic pathways. Thus the mouse model which expresses this type of fusion gene, in theory, will generate ES/PNET phenotype similar to human.

Chapter 4

EWS/FLI-1 Induced Transformation is Reversed by a KRAB/FLI-1 Suppressor Protein

4.1 Introduction

Transformation by EWS/FLI-1 is believed to be induced by its high ubiquitous expression and action as a potent transcription factor which dysregulates genes normally regulated by FLI-1 (Delattre *et al*, 1992; May *et al*, 1993a,b). Genes such as *c-myc*, *stromelysin-1*, *Manic Fringe*, *mE2C* and *EAT-2* etc. (Bailly *et al*, 1994; Braun *et al*, 1995; Thompson *et al*, 1996; May *et al*, 1997; Arvand *et al*, 1998) are associated with tumourigenesis in ES/PNET or EWS/FLI-1 transformed NIH3T3 cells. It has been suggested that these genes are normally regulated (directly or indirectly) by FLI-1 and are more efficiently activated by the *EWS/FLI-1* fusion gene, resulting in tumourigenesis. These downstream oncogenes, in turn, induce numerous tumourigenic pathways in ES/PNET. Several tumourigenic pathways have been shown to be activated by EWS/FLI-1 in ES/PNET including the IGF-IR signaling pathway (Toretsky *et al*, 1999), the Phospholipase C signaling pathway (Dohjima *et al*, 2000), the bFGF pathway (Girnita *et al*, 2000) and the mitogen-activated protein kinase (MAPK) signaling pathway (Silvany *et al*, 2000).

One approach to inhibit the transformed phenotypes of ES/PNET cells *in vitro* has been demonstrated by the removal or reduction of the expression of *EWS/FLI-1* transcripts through the introduction of antisense *EWS* fusion transcripts and oligonucleotides into ES/PNET cell cultures (Ouchida *et al*, 1995; Kovar *et al*, 1996; Tanaka *et al*, 1997; Toresky *et al*, 1997; Wang *et al*, 1999; Dohjima *et al*, 2000). This was associated with a reduction in the growth rate and reduced ability to form tumours in immuno-compromised mice (Ouchida *et al*, 1995; Kovar *et al*, 1996; Tanaka *et al*, 1997; Toresky *et al*, 1997). These approaches have been shown to significantly reduce the endogenous

EWS/FLI-1 transcripts in ES/PNET cells, inhibit the transformed phenotypes of ES/PNET and reduce activation of downstream signaling pathways (Ouchida *et al*, 1995; Kovar *et al*, 1996; Tanaka *et al*, 1997; Toretsky *et al*, 1997; Wang *et al*, 1999; Dohjima *et al*, 2000). These reports confirm that EWS/FLI-1 plays an important role in the tumourigenesis of ES/PNET. The wide variety of signaling pathways involved also indicate that this fusion protein activates a complex cascade of mediators which make understanding the aetiology and the clinical management of this disease difficult. Thus, the reduced expression of this fusion gene could be a potential therapeutic approach for elucidating the disease mechanism and clinical therapy for the Ewing family of tumours.

The low stability and efficiency of antisense transcripts/oligonucleotides *in vivo* make the therapeutic application of these approaches difficult (Jen *et al*, 2000). Therefore, an alternative approach to suppress the tumourigenic phenotype in ES/PNET was investigated in this study. This approach is involved the inhibition of the action of EWS/FLI-1 by introducing specific transcriptional suppressors into the ES/PNET cells that can bind to promoters and actively suppress EWS/FLI-1 target genes. Since the specificity of the DNA binding domain of EWS/FLI-1 appears to be an important determinant in the generation of ES/PNET (see Section 1.5), coupling of a potent suppressor domain, KRAB (Kruppel associated box), to the EDB domain of FLI-1 should target the suppressor to the same genes activated by EWS/FLI-1 in ES/PNET.

Recently, similar KRAB/DNA-binding-domain fusion proteins have been shown to efficiently suppress promoter-reporter constructs of *erbB-2/HER-2*, *HIV-1*, and *estrogen receptor* in a DNA binding specific manner (Beerli *et al*, 1998; Herchenroder *et al*, 1999; Ma *et al*, 1999; de Haan *et al*, 2000). These fusion proteins also appear to inhibit the transformed phenotypes of cell lines (Rossi *et al*, 1999; Fredericks *et al*, 2000; Nawrath *et al*, 2000; Ayyanathank *et al*, 2000).

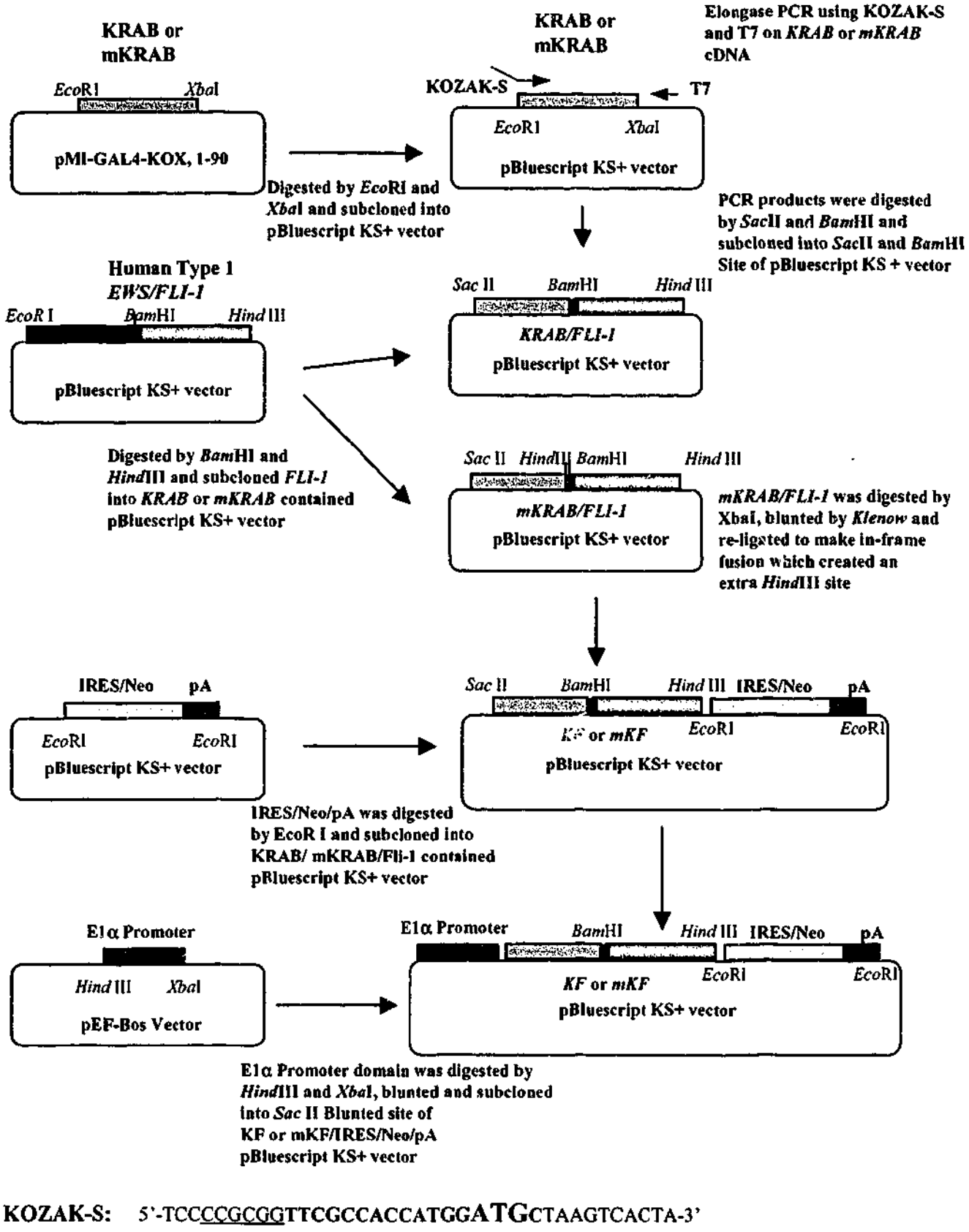
Thus, a specific transcriptional suppressor, KRAB/FLI-1 (KF), was engineered and its ability to reverse the phenotype of human ES/PNET cell lines or NIH3T3 cells transformed by mouse or human EWS/FLI-1 was investigated in this study. The ability of these cells to proliferate in low serum, form colonies in soft agar and tumours in nude

mice were examined to demonstrate that KF was able to suppress the transformed phenotypes of ES/PNET. Furthermore, the expression levels of *stromelysin-1*, *c-myc* and *EAT-2* were examined to show that the EWS/FLI-1 and KF fusion proteins modulate the transcription of specific genes. Moreover, since *stromelysin-1* has been shown most likely to be directly regulated by EWS/FLI-1 (Braun *et al*, 1995), this study also analyzed the *stromelysin-1* reporter constructs to determine whether this promoter fragment was regulated by EWS/FLI-1 and/or KF.

4.2 Results:

4.2-1 Construction of a KRAB/FLI-1 (KF) and mutant KRAB/FLI-1 (mutant KF) Transcriptional Repressors

The KRAB and mutant KRAB domains (amino acids 18 and 19 were changed from DV to AA) of KOX1 cDNA in pMI-GAL4-KOX vector (Fig.4.2) (kind gift from Drs. J Margolin and Frank J Rauscher III) were digested by *Eco*RI and *Xba*I and subcloned into pBluescript KS+ vector. The mutant KRAB was used as negative control because the two substitutions abolish the KRAB repressor function (Margolin *et al*, 1994). The *KRAB/mutant KRAB* cDNA sequences (amino acids 1-91) lacked an ATG-Kozak sequence that is required for the binding and assembly of the transcription initiation complex, leading to transcription in the mammalian system. Therefore, a KOZAK consensus sequence and initiation codon was added to *KRAB* and *mutant KRAB* by PCR amplification using the sense primer, KOZAK-S 5'-TCCCCGCGGTTCGCCACC**ATG**GATGCT-3' (Appendix D), T7 primer and *Elongase* polymerase (Life Technologies) in pBluescript KS+ vector. In this KOZAK-S primer, a *Sac* II site, KOZAK site and initiation site were designed (Fig.4.1 and 4.2). Thus, the PCR fragments were digested by *Sac*II and *Bam*H1 enzyme and subcloned into pBluescript KS+ vector (Fig.4.1). The human *FLI-1* DNA-binding domain (amino acids 220-452) was excised from human type 1 *EWS/FLI-1* cDNA (gift from Dr. O. Delattre) by *Bam*HI and *Hind* III with a small EWS cDNA fragment adjacent to FLI-1 joining point (amino acids 245-264) and fused to *KRAB/mutant KRAB* cDNA vectors at *Bam*HI and *Hind*III sites (Fig.4.1). This insertion caused in-frame fusion of *K/F*. The *mutant K/F* in-frame fusion needed one more *Xba*I digestion step which was blunted by *Klenow* later and re-ligated resulting an extra *Hind*III site (Fig.4.1). In order to have high expression of *KF* or *mutant KF* fusion gene in eukaryotic cells, the human E1α promoter from pEF-BOS vector was subcloned in front of *KF* or *mutant KF* fusion genes (Fig.4.1 and 4.2). A promoterless IRES/Neo/PolyA was cloned in the *Eco*R1 site behind the *KF or mutant KF* fusion gene to select for clones with high expression of *KF* or *mutant KF* under high concentrations of G418. The orientation and sequences of both



KOZAK-S: 5'-TCCCCGCGGTTCGCCACCATGGATGCTAAGTCACTA-3'
Sac II **KOZAK**

Fig.4.1 Schematic diagram showing steps to create *KF* or *mKF* fusion gene and their expression constructs. All constructs were confirmed for correct orientation and in-frame fusion by sequencing, restriction mapping, PCR analysis.

KRAB/FLI-1 and *mKRAB/FLI-1* Expression Constructs

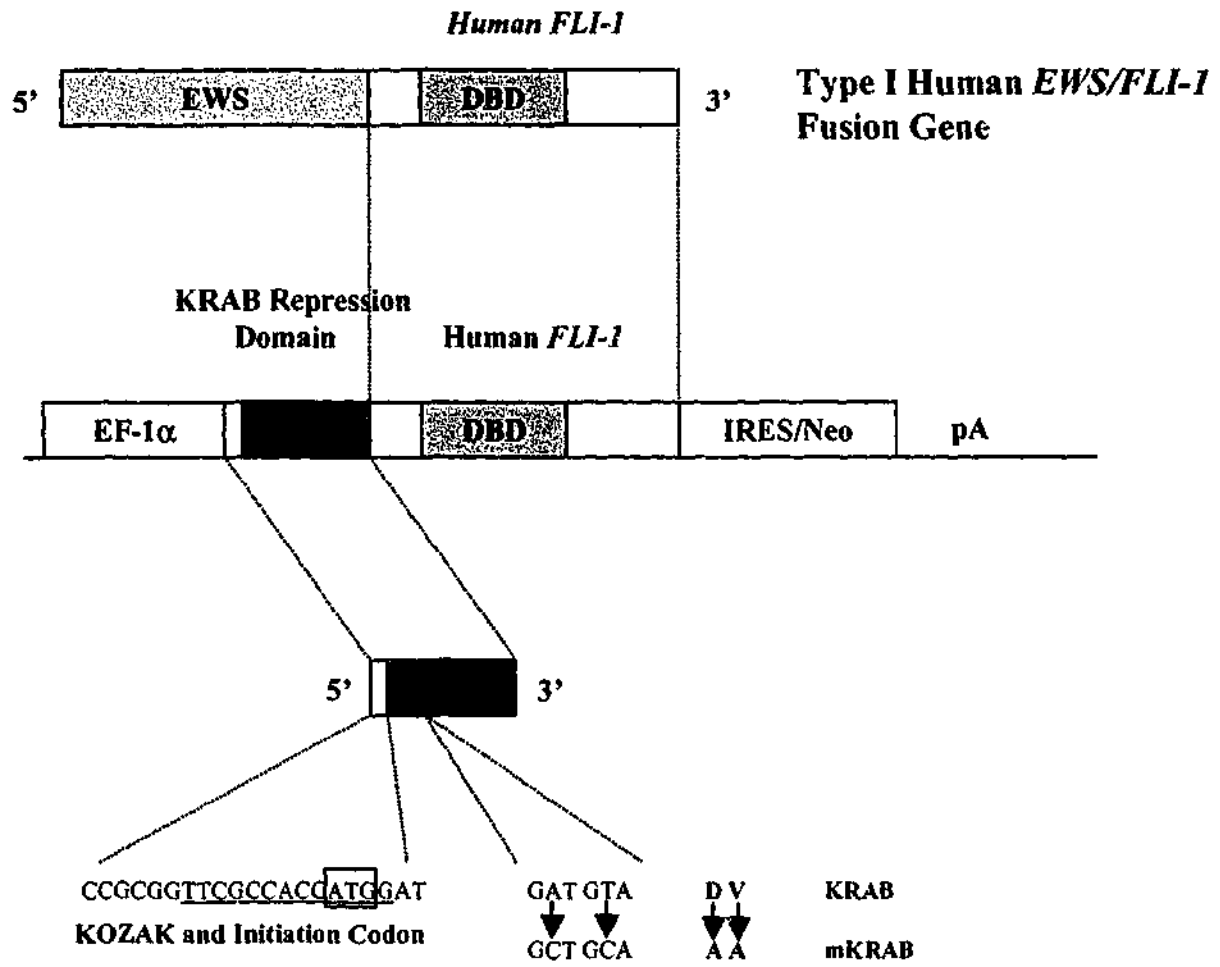


Fig.4.2 Schematic diagram of KF and mKF expression constructs. Either KRAB or mutant KRAB with an engineered KOZAK consensus sequence with initiation codon, ATG, was used to replace the EWS transactivation domain in the human Type I EWS/FLI-1 fusion gene. The KF or mKF with an IRES/Neo selection gene was driven by Elongation Factor-1α promoter.

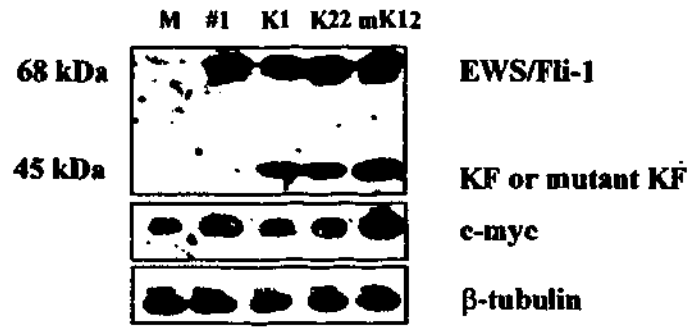
KF and *mutant KF* fusion genes were verified by restriction digestion and sequencing analysis.

4.2-2 Generation and Characterization of KF and mutant KF Transcriptional Repressors

Both KF and mKF expression constructs were linearised by *Sca*I and were electroporated into 5 murine and 5 human EWS/FLI-1 transformed NIH3T3 cell lines. They were also transfected into normal NIH3T3 cells which were used as controls. All transfected cell lines were selected on 400 μg G418 for 2 weeks. The G418-resistant colonies were cloned, expanded, cryopreserved and evaluated for expression of protein by Western analysis and RT-PCR.

The expression of KAP-1 gene in NIH3T3 cells is very important for the suppression effect of KRAB domain (Friedman *et al*, 1996; Peng *et al*, 2000a, b). This gene was identified in all murine and human EWS/FLI-1 transformed NIH3T3 cell lines and SK-N-MC, a human PNET cell line by RT-PCR using KAP-1S and KAP-1A primers which generates a 531 bp fragment (Appendix 4) (data not shown). To examine the expression of KF or mutant KF in murine or human EWS/FLI-1 transformed cell lines, a polyclonal antibody FLI-1 (C-19) (Santa Cruz Biotechnology, Inc. CA. Cat. # sc-356) was used in Western blotting. The KF or mutant KF positive cell lines express 45 kDa band (Fig.4.3). By RT-PCR, a 238 bp fragment was amplified by KRAB-SB 5' primer and a Fli-1-8A 3' primer (Appendix 4) (data not shown). All the KF or mutant KF positive clones shown by Western blotting were positive to RT-PCR analysis. Data from two representative murine and human EWS/FLI-1 transformed clones, mEF#1 and HuEF#16, transfected with *KF* are shown in Figure 3A & B. K1, K22 and mK12 are KF and mutant KF co-transfectants respectively of the mouse EWS/Fli-1 transformed clone mEF#1. These clones had similar EWS/Fli-1 protein levels to the parental mEF#1 relative to β-tubulin controls. mK12 displayed a slightly higher expression of mutant KF protein levels compared with K1 and K22 co-transfectants. Similar expression of human EWS/FLI-1, KF and mutant KF was observed in co-transfectants of the human EWS/FLI-1 transformed clone HuEF#16 (Fig.4.3). These data indicate that these

A **Murine EWS/Fli-1 Transformed Cell Line**



B **Human EWS/FLI-1 Transformed Cell Line**

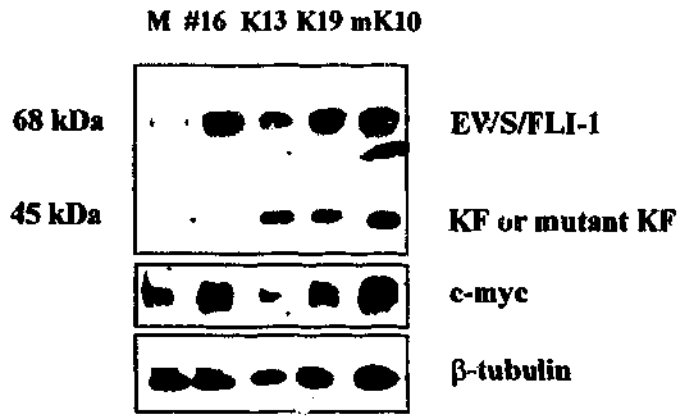


Fig.4.3 Western blot showing the expression of EWS/FLI-1 (68 kDa), KF or mutant KF (45 kDa) and alteration of c-myc protein levels in transfected NIH3T3 cells. (A) Cells transfected with empty construct (M = mock control) or murine EWS/Fli-1 (#1) and subclones of #1 co-transfected with KF (K1, K22) or mutant KF (mK12). (B) Similar human EWS/FLI-1 transformed clones transfected with KF (K13, K19) or mutant KF (mK16).

proteins are expressed at similar levels and thus any alterations observed in transformed phenotype of these clones is likely to be due to the function of these proteins rather than variable protein levels.

4.2-3 KF Reduces the Proliferation Rate of Murine and Human EWS/FLI-1 Transformed NIH3T3 Cells in Low Serum Media

When NIH3T3 cells were transfected by either murine or human EWS/FLI-1 fusion genes, they grow well in low serum medium (1% fetal calf serum) suggesting that they were transformed by EWS/FLI-1 (Fig. 3.2 C in Chapter 3). In this Chapter, KF was co-transfected into these EWS/FLI-1 expressing cells and expressing co-transfectants were found to have a significantly reduction in growth rate in low serum media. The mutant KF co-transfectants didn't show the same reduction in growth, suggesting that the KF specific transcriptional repressor has ability to inhibit EWS/FLI-1 transformation.

In a murine *EWS/Fli-1* expressing cell line mEF#1, the KF co-transfectants, mEF#1K1 and mEF#1K7, initially proliferated over the first 6 days and then the cell number declined. The growth rate of these two KF positive clones in mEF#1 was similar to normal controls (Fig. 4.4). However, the parental cell line, mEF#1, and mutant KF co-transfected mEF#1mK12 showed same growth rate which was faster than that of normal control cells and KF co-transfectants (Fig.4.4). In a human *EWS/FLI-1* expressing cell line HuEF#16, the KF co-transfectants, HuEF#16K13 and HuEF#16K19, displayed a similar significant reduction in growth rate compared with the parental transformed cell line HuEF#16 and the mutant KF co-transfectant (Fig.4.4). Thus, *KF* was able to inhibit the growth of both human and murine EWS/FLI-1 transformed cells. The KF transcriptional repressor reverses the effects of EWS/FLI-1 fusion protein. This leads NIH3T3 cells to be serum sensitive although the EWS/FLI-1 fusion gene is still expressed in these cells.

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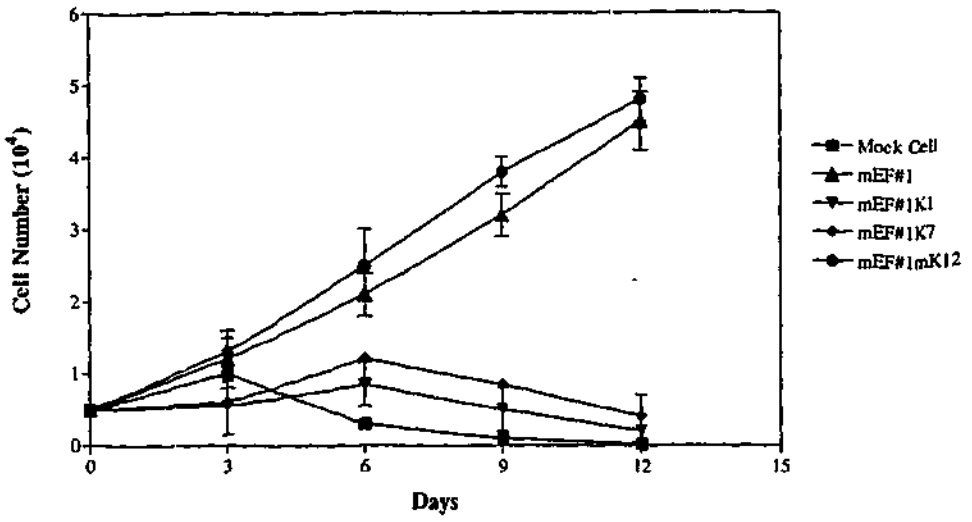

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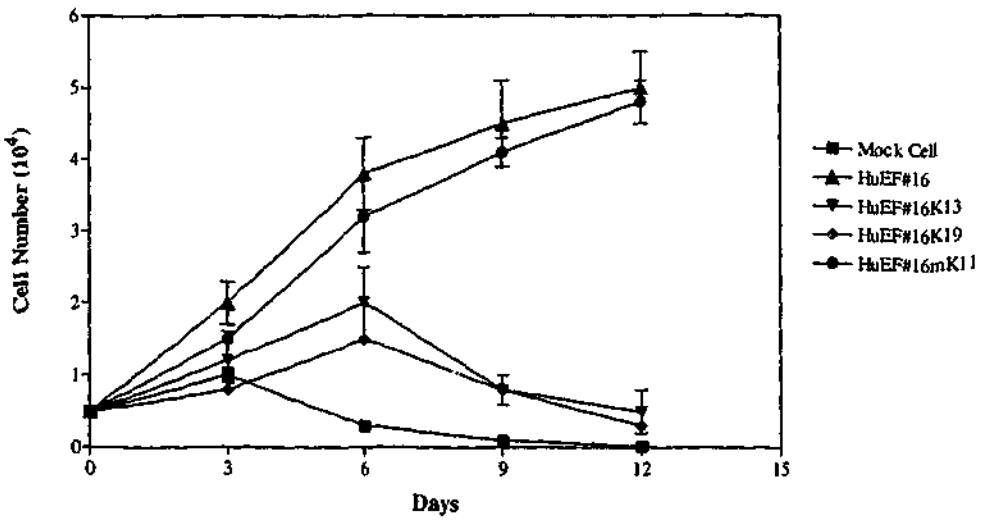

Fig.4.4 KF inhibits growth rate in low serum medium. Expansion of 5000 cells grown in 1% FCS. Cells were counted in triplicate at 3-day intervals and mean ±SEM is shown. (A) The parental murine EWS/Fli-1 transformed NIH3T3 cells (mEF#1) and KF (K1, K7) or mutant KF (mKF12) co-transfected subclones. (B) The parental human EWS/FLI-1 transformed NIH3T3 cells (HuEF#16) and KF-1 (K13, K19) or mutant KF (mK11) co-transfected subclones. NIH3T3 cells transfected with empty vector (M = mock control) were used as a control. Data is a representative of three separate experiments with mean (± SEM).

4.2-4 KF Reduces the Colony Formation of Murine and Human EWS/FLI-1 Transformed NIH3T3 cells in Soft Agar

Murine and human EWS/FLI-1 parental transformed cell lines, KF and mutant KF transfectants and normal control cell lines were seeded into soft agar media with complete growth medium containing 10%FCS. Each cell line was tested in triplicate in three separate experiments and colonies with >20 cells were scored. Data from two representative *EWS/FLI-1* expressing clones (one human, HuEF#16 and one murine, mEF#1) are shown in Figure 4.4. For the murine EWS/Fli-1 transformed cell line, mEF#1, clones which had high expression of KF such as mEF#1K1 and mEF#1K12 had significantly reduced colony formation in soft agar compared with the parental murine EWS/Fli-1 transformed cell line mEF#1 (Fig.4.5). Other clones with relatively low-levels of KF showed moderate inhibition of colony formation in soft agar media (data not shown). This correlates with previous data which suggested that the relative levels of KF to EWS/Fli-1 fusion proteins may affect the suppression of transformation ability in these cells. There was no significant inhibition of soft-agar growth in mutant KF co-transfectant mEF#1mK12 (Fig.4.5), suggesting that the suppression of KF was due to active suppression mediated by the KRAB domain and not due to inhibition of EWS/FLI-1 DNA binding. Similar to KF in murine EWS/Fli-1 transformed cells, the KF in human EWS/FLI-1 co-transfectants, HuEF#16K13 and HuEF#16K19 showed significant inhibition of colony-forming potential (Fig.4.5). There was no significant inhibition of soft-agar growth in the mutant KF transfectant, HuEF#16mK10 (Fig.4.5). The effect on inhibition of colony formation in KF transfectants also correlated with the reduced proliferation rate in low serum medium described above. These findings demonstrate that KF repressor causes impairment of anchorage-independent growth of EWS/FLI-1 transformed cells. This was also consistent with the notion that KF may repress some downstream target genes which have critical roles in EWS/FLI-1 tumourigenicity by interfering their transcriptional machinery.

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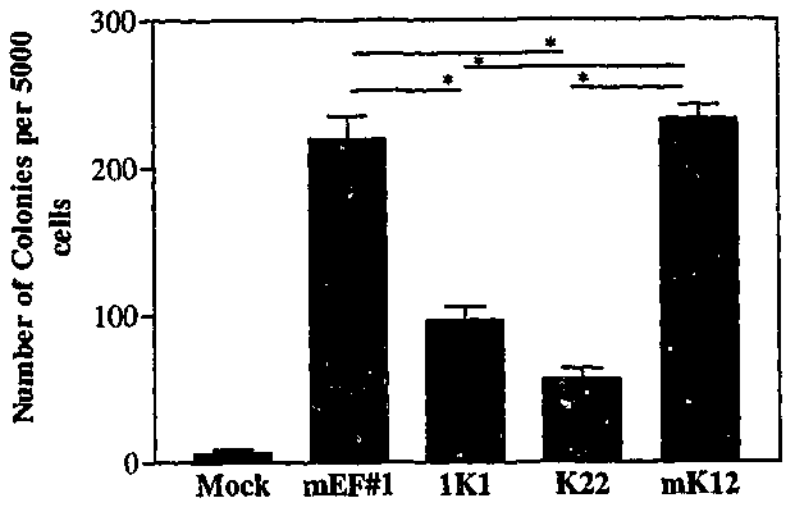

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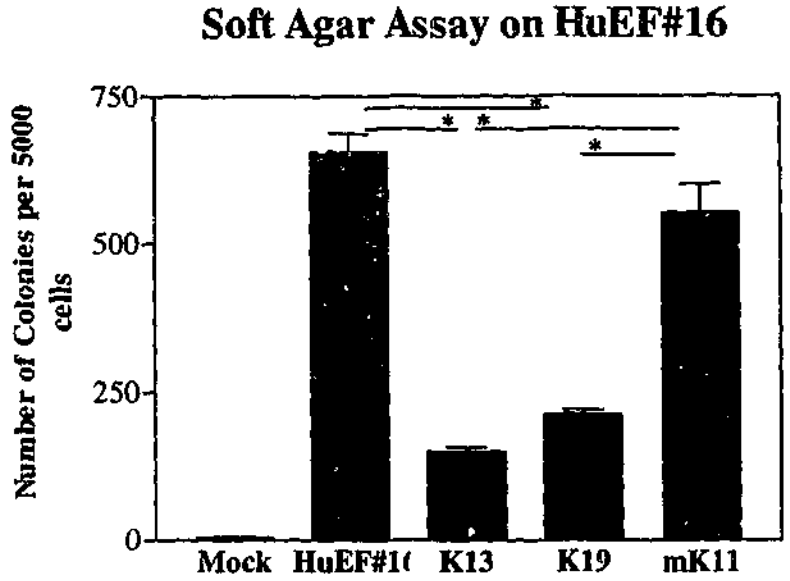


Fig.4.5 KF inhibits growth in soft-agar assays. (A) The parental murine EWS/Fli-1 transformed NIH3T3 cells (mEF#1) and KF (K1, K7) or mutant KF (mK12) co-transfected subclones. (B) The parental human EWS/Fli-1 transformed NIH3T3 cells (HuEF#16) and KF (K13, K19) or mutant KF (mK11) co-transfected subclones. Values shown are the mean $\pm$SEM of triplicate samples after 12 days. Data is a representative of three separate experiments. Samples indicated (*) were significantly different ($P \leq 0.05$).

4.2-5 KF Impairs Tumour Development of Murine and Human EWS/FLI-1 Transformed NIH3T3 Cells in Nude Mice

The above data showed a strong correlation between expression of KF and suppression of the EWS/FLI-1 transformed cells *in vitro*. In this study, the ability of KF to suppress tumour formation in nude mice by these EWS/FLI-1 transformed cells was determined. For each murine or human EWS/FLI-1 transformed clone, one KF co-transfectant and one mutant KF co-transfectant were inoculated into *BALB/c* nude mice. All the mice injected with the murine EWS/Fli-1 transformed cell line mEF#1 and the mKF co-transfectant mEF#1mK12 displayed large palpable tumours within 30 days (Fig.4.6). However, only 2 of 10 sites inoculated with the KF co-transfectant mEF#1K1 cell line displayed palpable tumour from day 33 (Fig.4.6). Even at day 50, only 5 out of 10 sites had developed small palpable tumours (Fig.4.6). The tumour volumes of the parental transformed cell line mEF#1 and the mKF co-transfectant, mEF#1mK12, indicated that they had similar growth rates (Fig.4.6). In contrast, the tumour volume observed was much smaller for the KF co-transfectant, mEF#1K1.

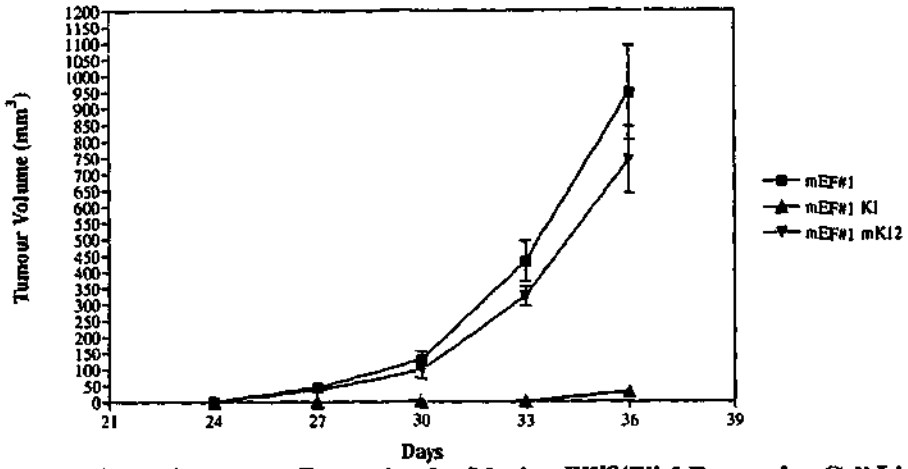
Similarly, for the human EWS/FLI-1 transformed NIH3T3 cell lines, all mice inoculated with the parental transformed cell line HuEF#16 or the mutant KF co-transfectant displayed grossly palpable tumours within 30 days (Fig.4.6). However, only 2 out of 10 sites inoculated with KF co-transfectant HuEF#16K13 displayed palpable tumours at day 33.

As a control, mock transfected NIH3T3 cells were subcutaneously injected into 4 *BALB*/c *nu/nu* female mice. Two out of 8 inoculation sites formed tumours at day 33. This indicated that a low level of spontaneous mutations may occur in these cells. However, this was significantly lower than that observed for EWS/FLI-1 transfectants.

These data also correlated with the proliferation rate in low serum medium and anchorage-independent growth in soft agar medium. This confirms that KF suppresses EWS/FLI-1 transformation of NIH3T3 cells. Since EWS/FLI-1 and KF act at the level of transcriptional activation, this also indicates that KF repressor protein may exert

A

Tumour Growth Rate in *BALB/c nu/nu* Mice
(Murine EWS/Fli-1 Transformed NIH3T3 Cells)

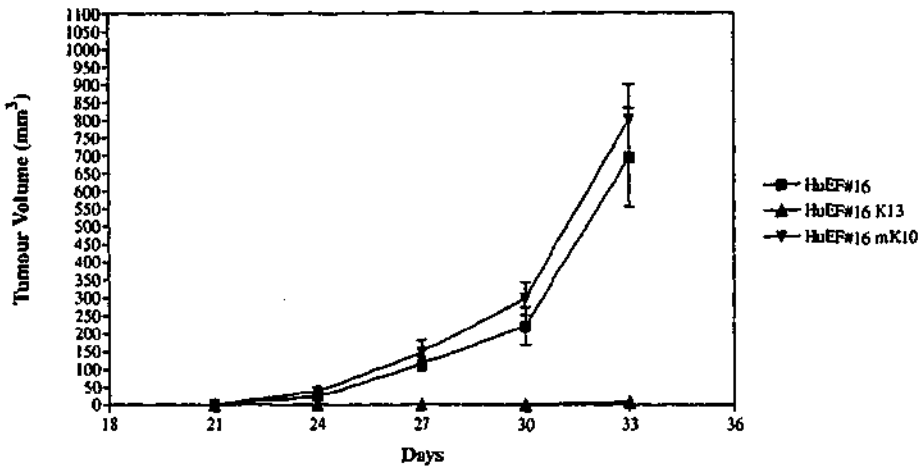


Number of Mice with Tumour Formation for Murine *EWS/Fli-1* Expressing Cell Lines

	Day 24	Day 27	Day 30	Day 33	Day 36
mEF#1	0/10	9/10	10/10	10/10	10/10
mEF#1 K1	0/10	0/10	0/10	2/10	5/10
mEF#1 mK12	0/10	6/10	9/10	10/10	10/10

B

Tumour Growth Rate in *BALB/c nu/nu* Mice
(Human EWS/FLI-1 Transformed NIH3T3 Cells)



Number of Mice with Tumour Formation for Human *EWS/FLI-1* Expressing Cell Lines

	Day 21	Day 24	Day 27	Day 30	Day 33
HuEF#1	0/10	7/10	8/10	8/10	10/10
HuEF#1 K1	0/10	0/10	0/10	2/10	5/10
HuEF#1 mK12	0/10	5/10	9/10	9/10	10/10

Fig.4.6 KF inhibits tumour growth in nude mice. 1 x 10^{6} cells were inoculated into the flasks of *BALB/c nu/nu* mice and tumours measured with calipers at 3-day intervals. (A) The parental murine EWS/Fli-1 transformed NIH3T3 cells (mEF#1) and KF (K1) or mutant KF (mK12) co-transfected subclones. (B) The parental human EWS/FLI-1 transformed NIH3T3 cells (HuEF#16) and KF (K13) or mutant KF (mK11) co-transfected subclones. The mean tumour volumes (± SEM) are shown and was calculated by the formula: $(\text{mean diameter})^3 \times \pi/6$. The table shows the number of inoculation sites forming tumours at each time point.

suppression effects on downstream targets activated by murine or human EWS/FLI-1 fusion proteins which in turn, revert the transformation phenotype.

4.2-6 Molecular Study on KF Suppression Effects on EWS/FLI-1 Tumourigenicity in NIH3T3 Cells

The above results show that the engineered KF repressor can suppress the tumourigenic phenotype of EWS/FLI-1 transformed NIH3T3 cells. However, it was unclear whether the molecular changes in NIH3T3 cells, associated with transformation induced by EWS/FLI-1 fusion protein, were suppressed by KF. Therefore, the levels of c-myc, stromelysin-1, manic fringe and EAT-2 genes in murine and human EWS/FLI-1 and KF co-transfected NIH3T3 cells were analysed by Western blot analysis, semi-quantitative PCR and real-time quantitative PCR in LightCycler PCR system.

4.2-6.1 Western Blot Analysis

By Western analysis, the Fli-1 (C-19) polyclonal antibody and c-myc polyclonal antibody (Santa Cruz Biotechnology Inc.) were used to show the EWS/FLI-1, KF, mutant KF and c-myc proteins of two selected murine and human EWS/FLI-1 transformed clones, mEF#1 and HuEF#16, and their KF/mutant KF co-transfectants. In this study, we collected the cell in 70% confluency of each clones for protein and total RNA extraction. Cell samples were collected from at least 3 individual pools.

The parental murine *EWS/Fli-1* transfected clone mEF#1 and the mutant KF co-transfectant mEF#1mK12 expressed higher level of c-myc than control cells (Fig.4.3). However, in KF co-transfectants, mEF#1K1 and mEF#1K22, the c-myc protein level was reduced and the levels were nearly as same as normal control cell line (Fig.4.3). Similarly, in human *EWS/FLI-1* transfected cell lines, the expression level of c-myc in both the parental HuEF#16 and the mutant KF co-transfectant HuEF#16mK10 was much higher than control cells (Fig.4.3). In KF co-transfectants, e.g. HuEF#16K1, the c-myc protein level was significantly repressed compared with parental HuEF#16

(Fig.4.3). This might indicate that the high expression level of KF fusion protein in this clone may result in greater repression of c-myc. These data also indicate that the reduction of c-myc expression level was due to the active suppressive effect of KF since clones expressing mutant KF had no change in the level of c-myc.

4.2-6.2 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was also used to measure the levels of EWS/Fli-1 putative downstream genes in this study. Total RNA of each cell lines was extracted from $1x10^6$ cells using High Pure RNA Isolation Kit (Roche) according to the instructions of manufacturer and quantified by spectrophotometer. The 1st strand cDNA of each cell lines was synthesis by Reverse Transcriptase Kit (Promega). The following primer pairs were used for PCR on each genes: *EWS/FLI-1*, EWS-7S and Fli-1 8A; *KF* or *mutant KF*, KRAB-SB and Fli-1 8A; *c-myc*, c-myc-S and c-myc-A; *EAT-2*, EAT-2S and EAT-2R; *GAPDH*, GAPDH-S and GAPDH-A (Appendix 4). The denaturation and amplification conditions were 94^0C for 30 seconds followed by 30 cycles of PCR. Each cycle of PCR included immediate denaturation at 94^0C for 30 seconds, 55^0C for 30 seconds and 72^0C for 1 minute. The annealing temperatures for each gene depended on the TM temperature of each primer pair.

After transfection of murine EWS/Fli-1 transformed cell lines, all cell lines showed the expression of *EWS/Fli-1* transcripts, in addition to *KF* transcripts in *KF* transfectants and *mutant KF* transcripts in *mutant KF* transfectants (Fig.4.7). Both mEF#1 and the mutant KF co-transfectant mEF#1mK12 expressed higher *c-myc* mRNA compared with normal control cell line (Fig.4.7). Conversely, in all *KF* transfectants of mEF#1 cell lines tested e.g. mEF#1K1, mEF#1K22 and mEF#1K7, the expression level of *c-myc* mRNA was repressed. In particular, the *c-myc* mRNA level was significantly reduced in mEF#1K1 and mEF#1K22 compared with the parental cell line, mEF#1 (Fig.4.7). This may be due to the relatively high level of KF in these two clones. Therefore the high expression level of KF appears to have a increased repressive effect on *c-myc* mRNA expression. These data were consistent with the protein levels showed by Western blot analysis.

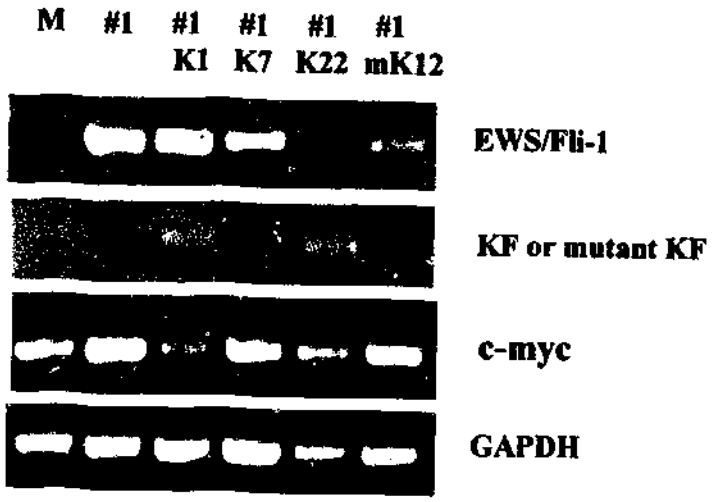


Fig.4.7 Semi-quantitative RT-PCR showing expression of *EWS/Fli-1*, *KF* or *mutant KF and c-myc* mRNA level in murine EWS/Fli-1 transformed cell lines.

For human EWS/FLI-1 transformed cell lines, both HuEF#16 and the mutant KF co-transfectant HuEF#16mK12 had higher transcription levels of the *c-myc* and *EAT-2* genes compared with the normal control (Fig.4.8). However, in two KF co-transfectants, HuEF#16K13 and HuEF#16K7, the mRNA expression levels of *c-myc* and *EAT-2* genes were greatly reduced (Fig.4.8). This demonstrates that the KF repressor can suppress EWS/FLI-1 downstream targets while for the mutant KF control, no suppressive effect was observed. The suppressive effect of KF was not only observed for *c-myc* mRNA expression levels but also for *EAT-2* mRNA level (Fig.4.8). These data indicate that the reversion of the tumour phenotype in these KF transfectants might due to suppression of downstream targets of EWS/FLI-1.

4.2-6.3 Real Time Quantitative RT-PCR

To evaluate the changes of mRNA expression level of these putative downstream target genes more accurately, a LightCycler system was applied to this study. In this system, the continuous fluorescence detection of amplifying cDNA allows rapid and accurate quantification of initial transcript amount. One step RT-PCR was performed with a LightCycler instrument (Roche) in a total volume of 20 μl containing 50 μg of total RNA, 3mM $MgCl_2$, 10 pmole each primer, LightCycler RT-PCR Reaction Mix SYBR Green I (1x) and LightCycler RT-PCR Enzyme Mix (Roche).

Quantitative analysis of the LightCycler data was performed employing LightCycler analysis software (Roche). To compensate for variability in RNA amount and for exclusion of general amplification effects, the initial template concentrations of *EWS/FLI-1* fusion gene, *KF* or *mKF* fusion genes and *c-myc* gene were normalised to those of an internal control, *GAPDH*. Results are reported as calculated in Genes/GAPDH ratio value. At least three experiments on three independent total RNA pools of each cell line are shown in Fig.4.9 and Fig.4.10.

For murine EWS/Fli-1 transformed clones, the quantitative RT-PCR data showed the expression levels of *EWS/Fli-1* were similar in the parental line mEF#1, *KF* co-transfectants mEF#1K1, and *mutant KF* co-transfectant mEF#1mK12 (Fig4.9). In *KF*

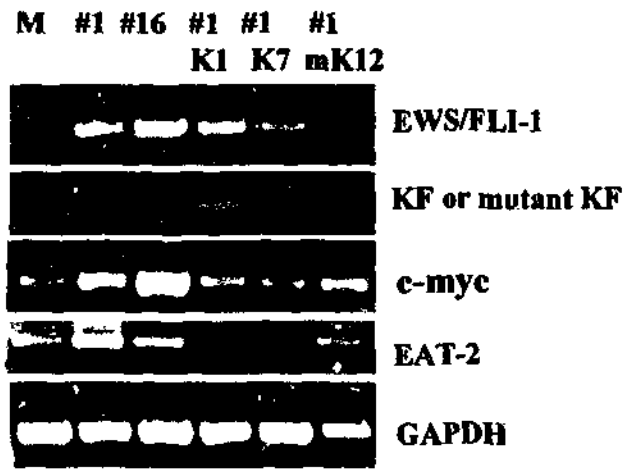


Fig.4.8 Semi-quantitative RT-PCR showing expression of *EWS/FLI-1*, *KF* or *mutant KF*, *c-myc* and *EAT-2* mRNA level in human EWS/FLI-1 transformed cell lines.

and *mutant KF* co-transfectants, *KF* and *mutant KF* were also expressed at similar levels (Fig.4.9). In contrast, the expression levels *c-myc* mRNA were significantly increased in EWS/Fli-1 transformed clones, but this effect was significantly reduced in co-transfectants expressing *KF* (Fig.4.9). The EWS/Fli-1 transformed cells transfected with *mutant KF* (mEF#1mK12) did not have reduced *c-myc* levels (Fig.4.9). These data were consistent with the Western blot and semi-quantitative RT-PCR analysis in this study.

In human EWS/FLI-1 transformed cell lines, the mRNA expression levels of EWS/FLI-1 were more variable (Fig.4.10). However, for clones such as HuEF#16K13 and HuEF#16mK11, which have similar levels of *EWS/FLI-1* mRNA expression levels, the *c-myc* mRNA expression level was significantly lower in HuEF#16K13 but not HuEF#16mK11 (Fig.4.10). Therefore, these data confirm that the KF repressor is capable of suppressing downstream genes of *EWS/FLI-1*. The suppression of these genes to normal levels correlated with the inhibition of EWS/FLI-1 induced transformation events in NIH3T3 cells.

4.2-7 Transfection and Expression of KF or mKF Fusions in Human PNET Cell Line, SK-N-MC

The human PNET cell line, SK-N-MC, has a Type 1 *EWS/FLI-1* fusion gene resulting from a chromosomal translocation, t(2;11;22;21)(q32;q24;q12;p11) (Giovanini *et al*, 1994). These cells are p53 deficient (Chen *et al*, 1995; Darnell *et al*, 1999) but express KAP which is required for the suppression effect of the KRAB domain (data not shown). Thus this cell line was used to study the suppression effects of KF repressor and to compare the suppression effects found in NIH3T3 cells. Similar to the electroporation processes in EWS/FLI-1 transformed cells, both *KF* and *mutant KF* expression constructs were linearised by *Sca*I and were electroporated into SK-N-MC cell line. An empty vector was transfected into SK-N-MC which as a control. All transfected cell lines were selected for 400 μg G418 resistance for 2 weeks. Resistant clones were carefully picked and isolated in 24-well plates. The individual clones was expanded and characterized by Western analysis and RT-PCR.

Murine EWS/Fli-1 Transformed Cell Lines

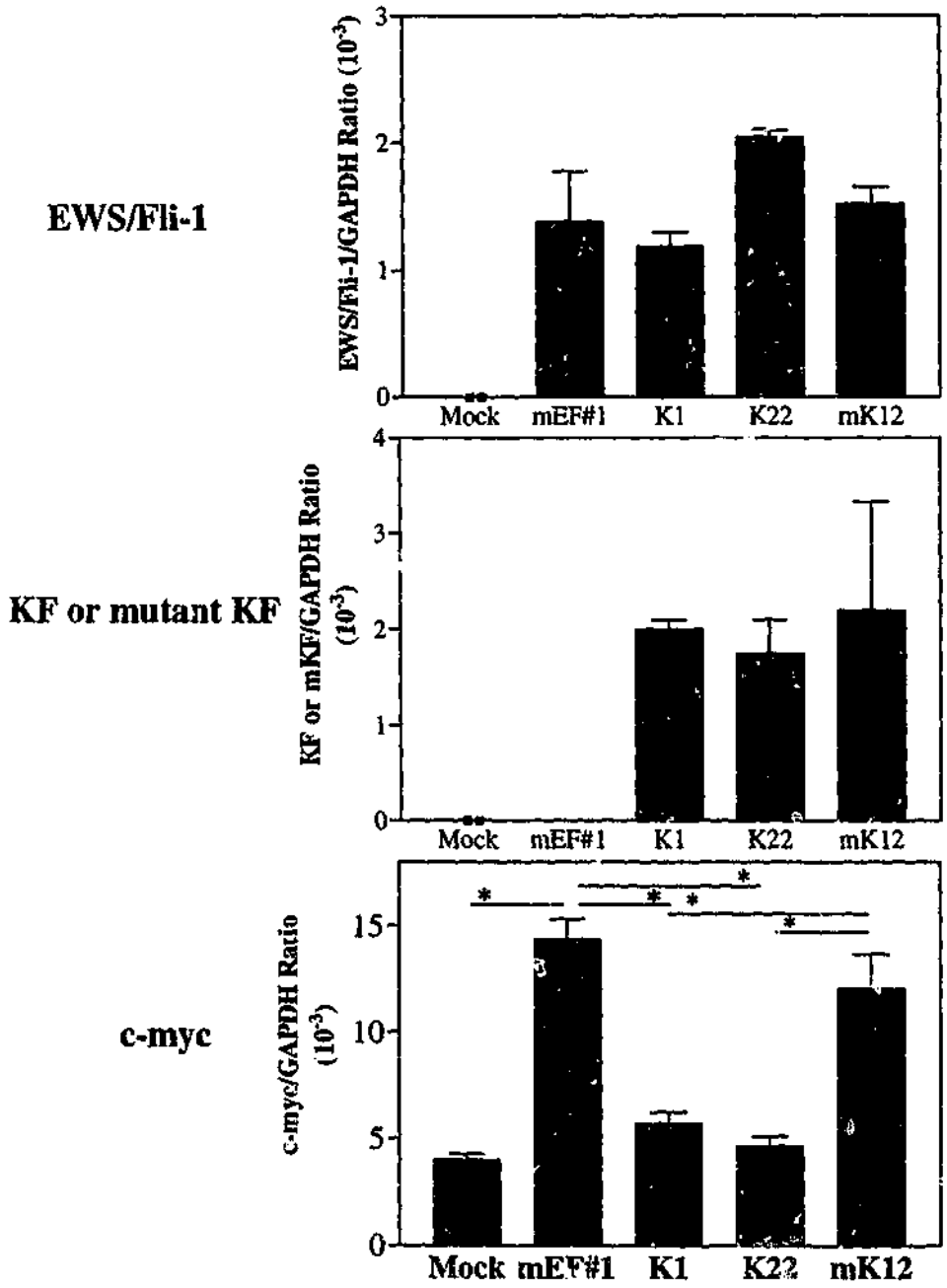


Fig.4.9 KF represses the *c-myc* transcriptional level. Expression levels of *EWS/Fli-1*, *KF* or *mutant KF*, and *c-myc* in the parental murine EWS/Fli-1 transformed NIH3T3 cells (mEF#1) and KF (K1, K22) or mutant KF (mK12) co-transfected subclones were determined by real-time RT-PCR using the lightCycler system. All data was normalized to *GAPDH* mRNA levels and shown as mean (±SEM) data is the mean of three experiments and samples indicated (*) were significantly different ($P \leq 0.05$).

Human EWS/FLI-1 Transformed Cell Lines

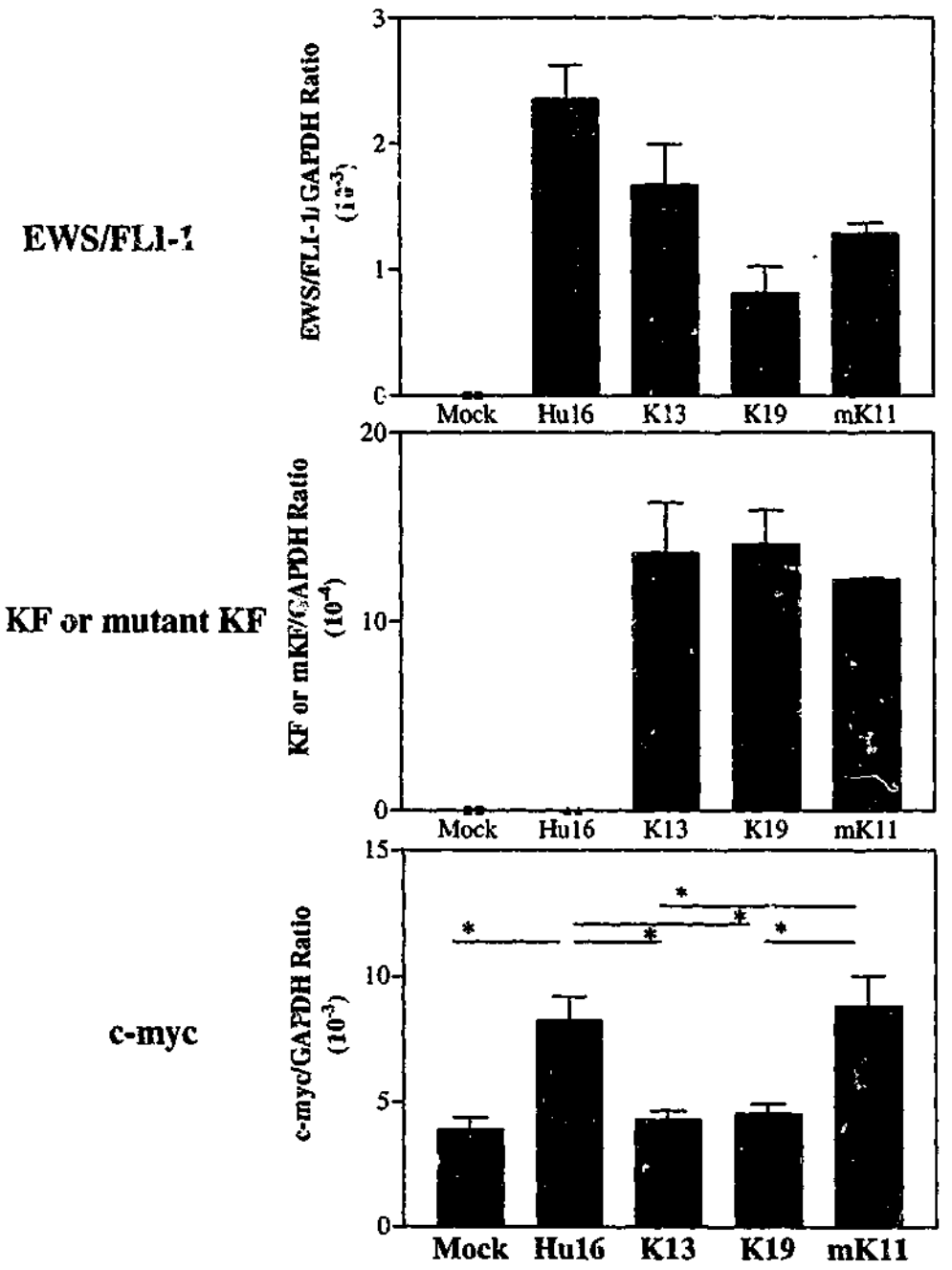


Fig.4.10 KF represses the *c-myc* transcriptional level. Expression levels of *EWS/FLI-1*, *KF* or *mutant KF*, and *c-myc* in the parental human EWS/FLI-1 transformed NIH3T3 cells (HuEF#16) and KF (K13, K19) or mutant KF (mK11) co-transfected subclones were determined by real-time RT-PCR using the lightCycler system. All data was normalized to *GAPDH* mRNA levels and shown as mean (±SEM) data is the mean of three experiments and samples indicated (*) were significantly different ($P \leq 0.05$).

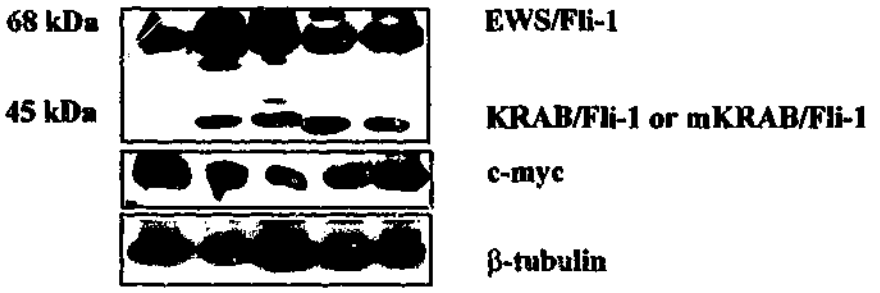
The Fli-1 polyclonal antibody (Santa Cruz) identified the 68 kDa EWS/FLI-1 fusion protein in all cell lines and the 45 kDa KF and mutant KF in *KF* and *mutant KF* co-transfectants respectively (Fig.4.11). The SKM clone represented the mock transfected control of SK-N-MC with empty vector. Two *KF* transfectants and two *mutant KF* transfectants were randomly selected for extensive analysis of KF repressor function. Western analysis demonstrated that the EWS/FLI-1 fusion protein was expressed at a much higher level than the levels of KF or mutant KF fusion protein. This correlated with the observation that the EWS/FLI-1 expression was elevated in the cells exhibiting rapid growth (Tanaka *et al*, 1997). By using semi-quantitative RT-PCR, the expression levels of *EWS/FLI-1*, *KF* and *mutant KF* mRNA were also determined. These expression levels correlated with the Western blot analysis demonstrating that EWS/FLI-1, KF and mutant KF were all expressed although there was variation in the ratio of EWS/FLI-1 to KF and mutant KF between different subclones (Fig.4.11).

4.2-8 The KF Repressor Reverses the Transformed Phenotype of the Human PNET Cell Line, SK-N-MC

4.2-8.1 Reduce Proliferation Rate and Altered Morphology in Low Serum Media

Although the expression of EWS/FLI-1 fusion protein was much higher than KF protein in the SK-N-MC transfected cells, the KF repressor was still capable of suppressing the tumourigenic phenotype. This cell line grew rapidly in normal medium (10% FCS), low serum medium (1% FCS) and serum free medium (data not shown). After transfection of the *KF* fusion gene into this cell line, the morphology and proliferation rate of *KF* transfected cell lines was unaltered in normal medium (data not shown). However, when cultured in low serum medium, the *KF* transfectants (e.g. SK-K15 and SK-K16) had a significant reduction in proliferation rate compared to the mock control (Fig.4.12). This suggests that SK-N-MC cell proliferation becomes sensitive to serum growth factors in the presence of KF repressor. On the other hand, the *mutant KF* transfectants (e.g. SK-mK3 and SK-mK15) did not show any reduction of proliferation rate (Fig.4.12),

A



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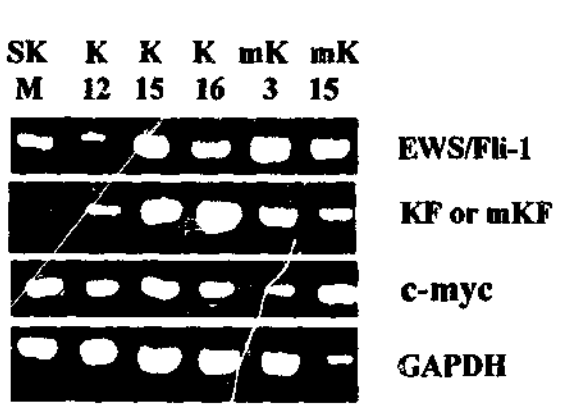


Fig.4.11 KF inhibits transformed phenotype of a human PNET cell line (A) Western blot showing the expression of EWS/FLI-1, KF or mutant KF and change of c-myc protein levels. (B) Semi-quantitative RT-PCR showing the expression of *EWS/FLI-1*, *KF*, *mutant KF* and the change of *c-myc* mRNA levels.

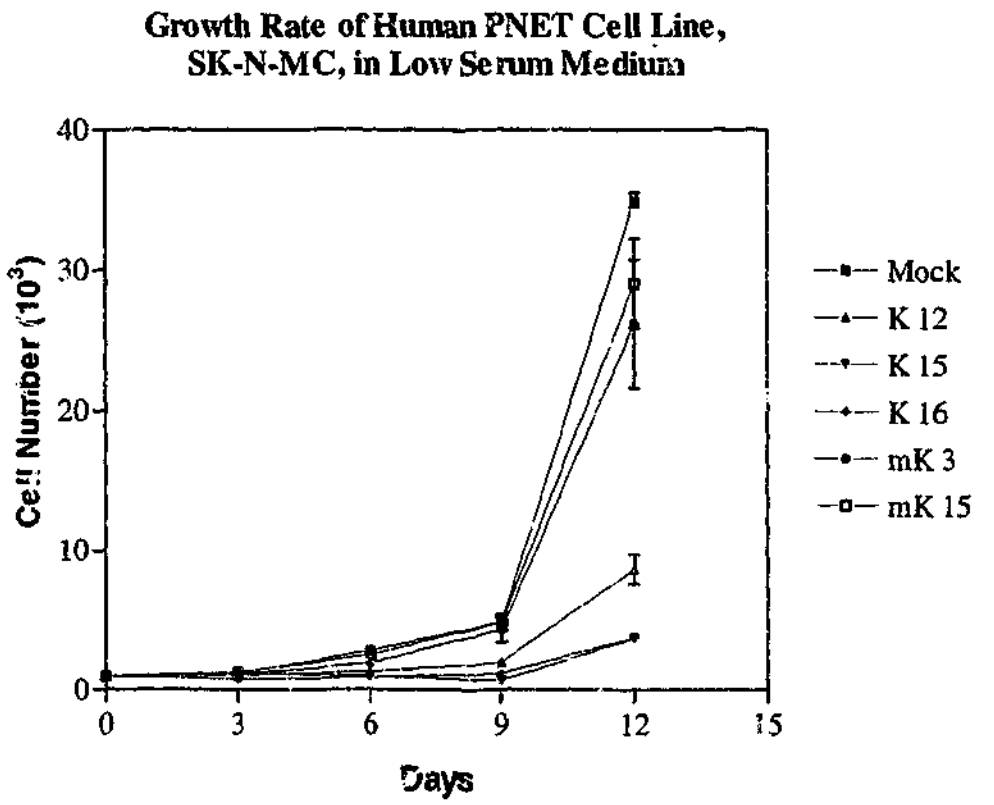


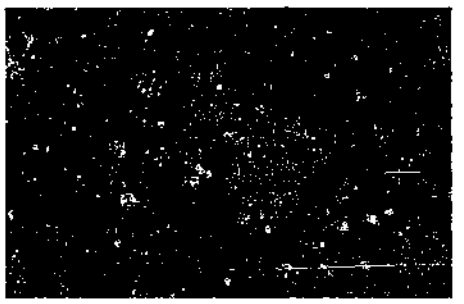
Fig.4.12 KF inhibits growth rate of a human PNET cell line in low serum media. Mean ±SD of 1×10^3 cells seeded in triplicate in media supplemented with 1% FCS at 3-day intervals. Mock = empty vector, K12, K15 and K16 were *KF* expressing clones while mK3 and mK15 were *mutant KF* expressing clones.

suggesting that the reduction of proliferation rate in SK-N-MC was due to the suppression effects exerted from the active KRAB domain in KF.

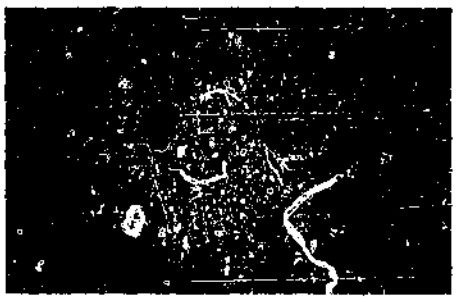
The SK-N-MC, mock control, *KF* or *mutant KF* transfectants grew as adherent cell clusters which expanded quickly in normal serum medium (data not shown). In low serum medium, the mock and mutant KF cells displayed similar morphology (Fig.4.13). However, the KF transfected cells (e.g. SK-K15 and SK-K16) rounded up and clusters were less compacted under low serum conditions (Fig.4.13). The cell numbers of each cluster was significantly reduced compared with the mock or mKF transfectants (Fig.4.13). This morphology was similar to that of cells undergoing apoptosis. Hence, the KF could efficiently reverse the serum growth factor independent-growth of SK-N-MC cells.

4.2-8.2 Suppression of Tumour Formation in Nude Mice

The above results suggest a correlation between the expression of KF and suppression of the growth of SK-N-MC cells *in vitro*. To further investigate the suppression of KF on the tumourigenic potential of SK-N-MC, the ability of these cells to develop tumours in nude mice was examined. Five *BALB/c nu/nu* female mice were subcutaneously injected with a SK-N-MC mock transfected cell line, a *KF* transfectant (SK-K16) and a *mutant KF* transfectant (SK-mK15). For each cell line, $5x10^6$ cells per 200 µl was subcutaneously injected into two inoculation sites of each nude mouse. Seven out of 10 inoculation sites of SK-N-MC mock transfected cells displayed palpable tumours by day 6 (Fig.4.15). At day 9, all sites inoculated with SK-N-MC control cells had formed grossly palpable tumours (Fig.4.15). The *mutant KF* transfectant, SK-mK15, had similar tumour formation compared to SK-N-MC mock cells indicating that the mutant KRAB domain did not suppress tumour formation or growth (Fig.4.15). However, the *KF* transfectant clone, SK-K16, formed tumours at only 2 out of 10 sites at day 6 (Fig.4.15). Even at day 18, significantly fewer sites (5 out of 10 sites) had developed small palpable tumours (Fig.4.15). Thus a smaller proportion of sites inoculated with *KF* transfectants developed tumours compared with SK-N-MC mock or *mutant KF* transfectants.

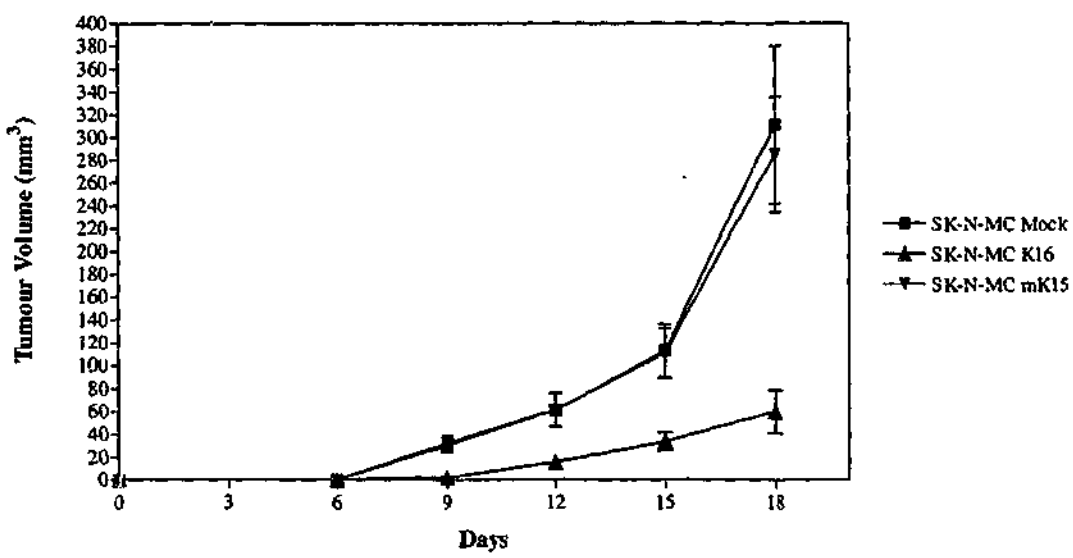


KF transfected SK-N-MC cells



Mutant KF transfected SK-N-MCcells

Fig.4.13 Cell colony morphology of KF and mutant KF transfected SK-N-MC cell lines in low serum medium.



Number of Mice with Tumour Formation for Human PNET Cell Line, SK-N-MC

	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
SK-N-MC Mock	0/10	7/10	10/10	10/10	10/10	10/10
SK-N-MC K16	0/10	2/10	3/10	3/10	5/10	5/10
SK-N-MC mK15	0/10	6/10	10/10	10/10	10/10	10/10

Fig.4.15 KF inhibits the tumour growth rate of a human PNET cell line *in BALB/c nu/nu* mice. 1 x 10^5 cells were inoculated into the flasks of *BALB/c nu/nu* mice and tumours measured with calipers at 3-day intervals. Mean tumour volumes (± SEM) are shown and were calculated by the formula: (mean diameter)^3 x $\pi/6$. The table shows the number of inoculation sites forming tumours at each time point.

The tumour growth rate was also significantly slower for the *KF* transfectant SK-K16. This clone was almost five-fold slower than SK-N-MC mock and *mutant KF* transfectants (Fig.4.15). However, *KF* expressing SK-N-MC cells formed tumour faster than NIH3T3 cells expressing both *KF* and *EWS/FLI-1*, suggesting that this level of the KF repressor could not completely abolish the transformed phenotype of SK-N-MC cells. This may be due to the relative levels of KF and EWS/FLI-1 proteins or the absence of p53 and other gene deletions in SK-N-MC cells. These *in vitro* and *in vivo* results indicate that there is a strong suppression effect of the KF in SK-N-MC cells that could decrease the number and size of tumours formed in nude mice.

4.2-9 Modulation of Putative Downstream Target Genes by KF Repressor in SK-N-MC Cells

The *c-myc* gene is a good molecular indicator of transformation which correlated with the cell proliferation rate and alterations in suppression effects of KF in murine or human EWS/FLI-1 transformed NIH3T3 cells. To determine whether *c-myc* levels correlated with KF induced suppression on tumourigenic phenotypes in SK-N-MC, the alterations of *c-myc* gene in SK-N-MC mock transfected cells, KF and mutant KF transfectants were evaluated by Western blot analysis, semi-quantitative RT-PCR and real time quantitative RT-PCR. Western blot analysis was performed using the c-myc polyclonal antibody (Santa Cruz) on cell extracts of each cell line. These data showed no significant reduction in c-myc protein expression level in KF transfectants (e.g. SK-K15 and SK-K16) unlike the repression effects observed with KF in murine or human EWS/FLI-1 transformed NIH3T3 cells (Fig.4.11). This may be due to the relative higher expression of EWS/FLI-1 fusion protein compared to KF repressor in SK-N-MC cells. This was supported by the observation that in a KF transfected clone, SK-K16, which had relatively higher expression of KF, the c-myc protein level was slightly reduced (Fig 4.11). Moreover, by semi-quantitative RT-PCR and real time quantitative RT-PCR, the *KF* mRNA was higher in SK-K16 compared with other clones and was associated with a significant reduction of *c-myc* transcripts (Fig.4.11 and Fig.4.14). The levels of *c-myc* transcript production were not affected by culture in complete or low serum media. Therefore, the KF repressor suppressed the transformed phenotype of SK-N-MC cells similar to that observed in EWS/FLI-1 transformed NIH3T3 cells. The

Human PNET cell line, SK-N-MC

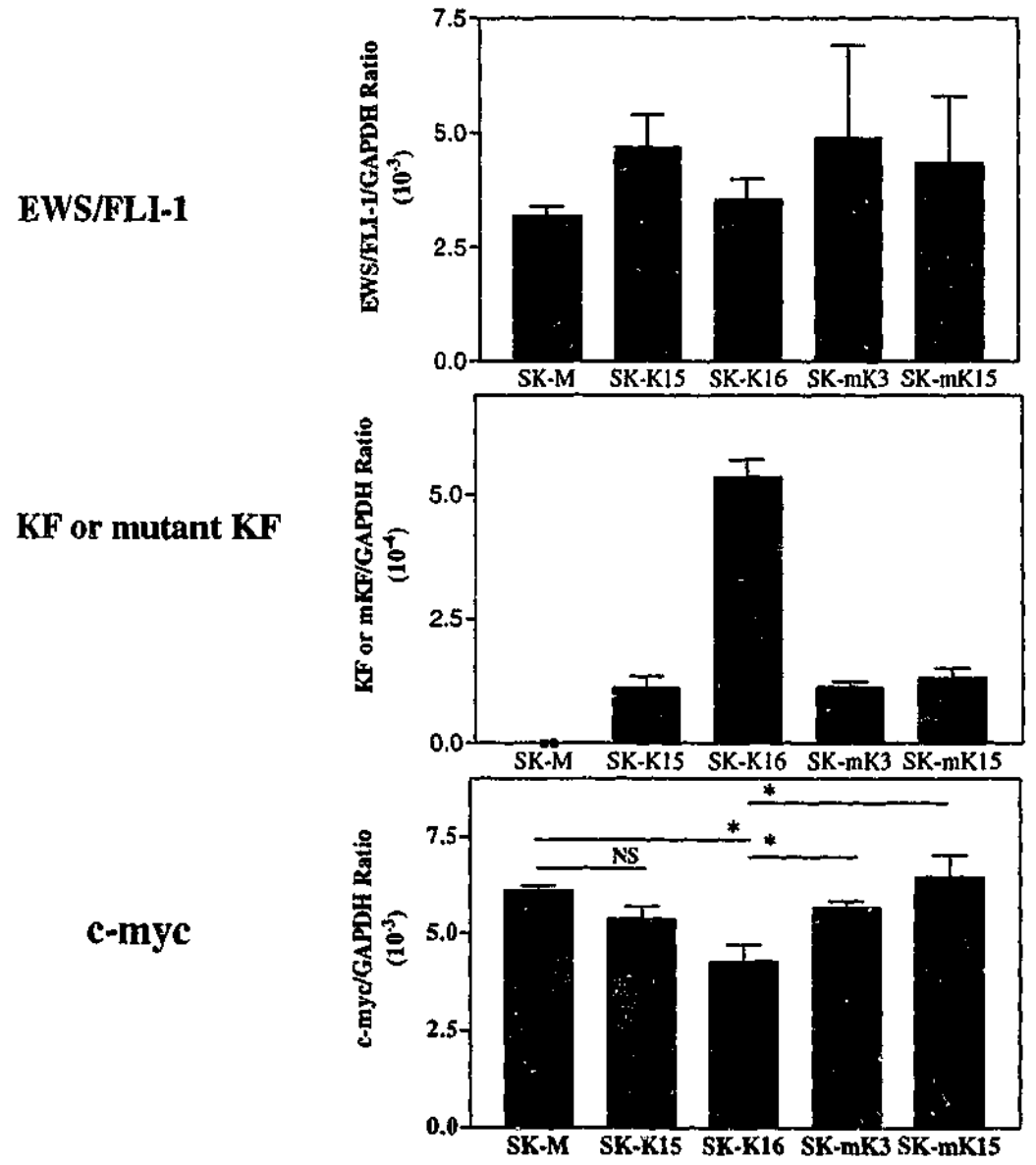


Fig.4.14 KF represses the *c-myc* transcriptional level. The mRNA expression levels of *EWS/Fli-1*, *KF* or *mutant KF* and *c-myc* analysed by quantitative RT-PCR. All data was normalized to *GAPDH* mRNA levels and are shown as mean ±SEM of three experiments. Samples indicated (*) were significantly different ($P \leq 0.05$).

higher expression of EWS/FLI-1 in SK-N-MC may explain the less efficient suppression of both the phenotype and c-myc levels in these cells.

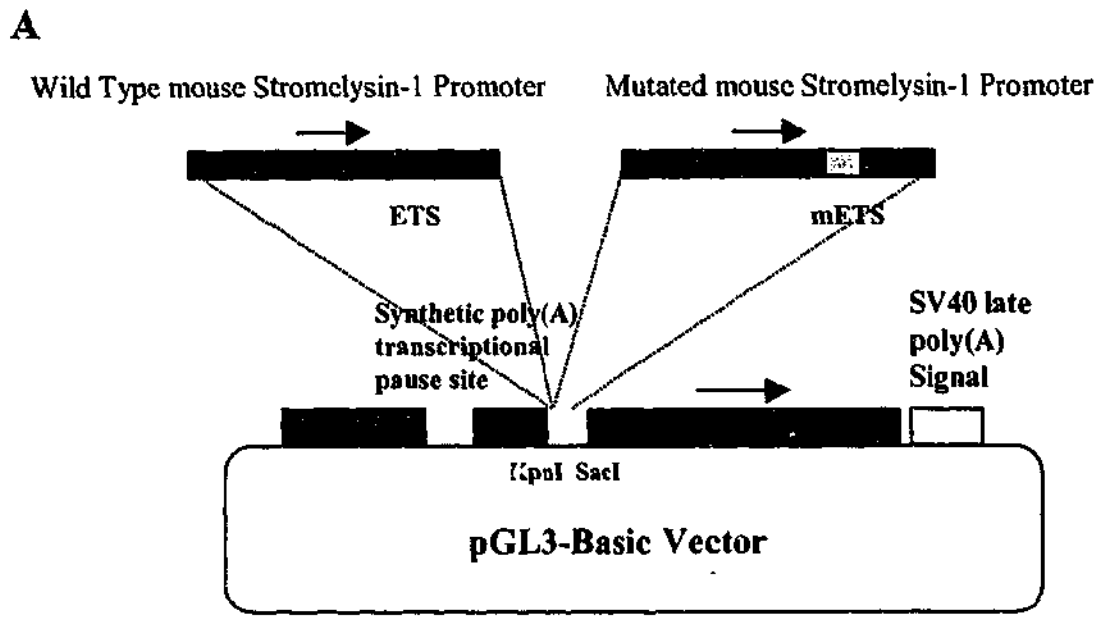
4.2-10 Investigation of Primary Targets of EWS/FLI-1 and KF

The *stromelysin-1* gene has been shown to be up-regulated by EWS/FLI-1 (Braun *et al*, 1995) and the promoter of this gene has been shown to contain two tandem ETS binding motif that are responsive to ETS-1, ETS-2 and TEL (Wasylyk *et al*, 1991, 1992; Fenrick *et al*, 2000). Since the DNA binding sequence is highly conserved between FLI-1 and ETS1 or ETS2, *stromelysin-1* is potentially a direct target gene of EWS/FLI-1 and KF. Thus, the responses of a *stromelysin-1* promoter reporter construct were examined in the *EWS/FLI-1* and *KF* transfected cell lines.

A 1.4kb fragment of the mouse *stromelysin-1* promoter sequence (Accession number: AF077676) was amplified by *Elongase* PCR. This fragment was cloned into a luciferase reporter vector, pGL3-Basic vector, at *Kpn*I and *Sac*I sites (Fig.4.16). To examine the putative EWS/FLI-1 binding site on this mouse stromelysin-1 promoter, this ETS binding site (-229 to –225; ggcaGGAAgcat) was mutated (ggcaTGCAgcat) by an overlapping PCR approach. The wild-type mouse *stromelysin-1* promoter luciferase reporter construct was designated as St-Luc while the mutated mouse *stromelysin-1* promoter luciferase reporter construct was designated mSt-Luc. The parental human EWS/FLI-1 transformed NIH3T3 cell line, HuEF#16, murine EWS/Fli-1 transformed cell line, mEF#1 and SK-N-MC were used for transient transfection with various combinations of KF, mutant KF, St-Luc and mSt-Luc. Transfections were performed using Lipofectamine 2000 reagent (Life Technologies) according to manufacturer's instructions. All transfections were performed in triplicate and each DNA construct was analysed in at least three independent exp iments. Forty-eight hours post transfection cells were lysed and assayed for luciferase (Promega) according to manufacturer's instructions.

Transfection of St-Luc into mEF, HuEF NIH3T3 transfectants and SK-N-MC cells resulted in a dramatical increase in the relative luciferase activity compared with pGL3-Basic vector (18.7 folds in mEF#1, 13.9 folds in HuEF#16 and 75.1 folds in SK-N-MC)

(Fig.4.16). However, when the mSt-Luc was transfected into these cells, the relative luciferase activity was not significantly different from the St-Luc (15.8 folds in mEF#1, 11.4 folds in HuEF#16 and 53.8 folds in SK-N-MC) (Fig.4.16). This indicated that mutation of one ETS binding site on 1.4 kb mouse *stromelysin-1* promoter was not enough to abolish the EWS/FLI-1 activity. Furthermore, when the St-Luc transfected into KF and mutant KF co-transfectants of mEF, HuEF and SK-N-MC cells, the relative luciferase activity was not significantly different (15.5 folds in mEF#1K1 and 12.5 folds in mEF#1mK11 of mEF#1 KF and mutant KF co-transfectants respectively; 8.1 folds in HuEF#16K13 and 10.9 folds in HuEF#16mK10 of HuEF16 KF and mutant KF co-transfectants respectively; and 66.1 folds in SK-K6 and 40.1 folds in SK-mK15 of SK-N-MC KF and mutant KF co-transfectants respectively) (Fig.4.16). These data indicated that the 1.4 kb mouse *stromelysin-1* promoter did not contain all the regulatory elements required for the altered transcription of this gene observed in all the transfectants of this study and in Ewing's sarcoma cells.



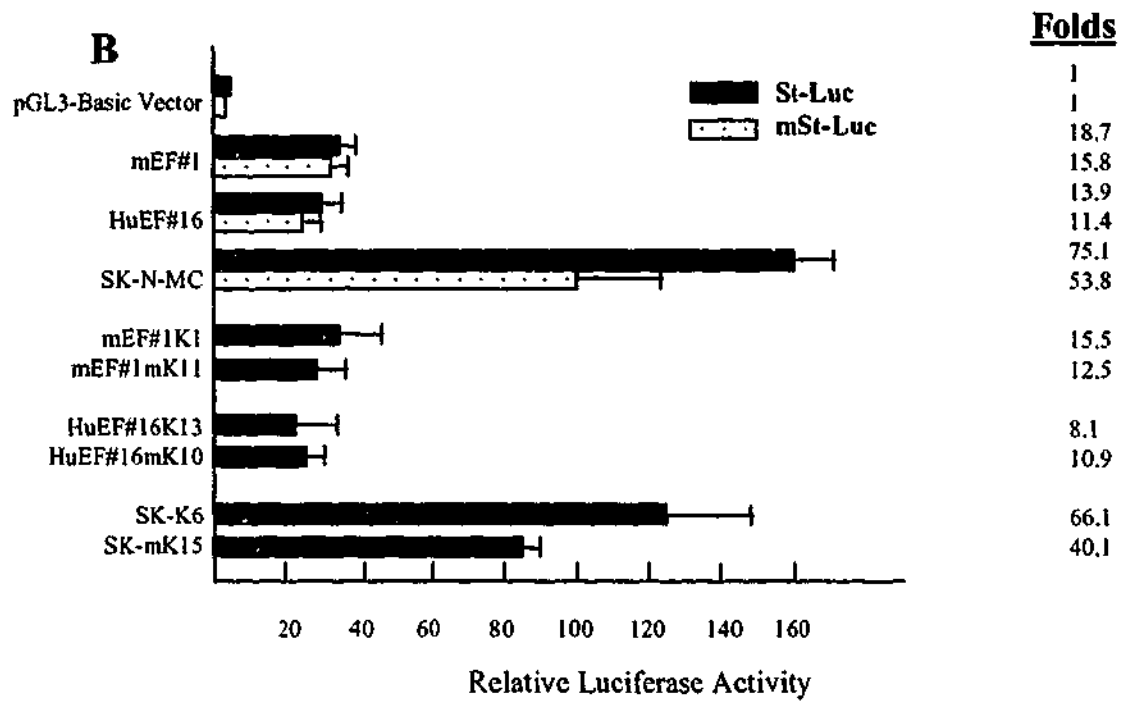


Fig.4.16 EWS/Fli-1 or KF binding and transcriptional activity on mouse *stromelysin-1* promoter using luciferase reporter construct. (A) Schematic diagram showing the cloning of wild type and mutated mouse stromelysin-1 promoter into pGL3-Basic, luciferase reporter vector. One putative ETS binding site at 229 to 225 was mutated into mETS. (B) The relative luciferase activity (mean ± S.D.) of the transient transfection of St-Luc and mSt-Luc reporter vectors in mEF, HuEF, SK-N-MC cells and their KF and mutant KF co-transfectants. The number of folds was calculated by compared the relative luciferase activity of St-Luc and mSt-Luc of each cell lines to that of pGL3-Basic Vector control respectively.

Discussion:

Suppression of ES/PNET cell growth has been attempted by a number of mechanisms, including antisense and dominant negative approaches to reduce EWS/FLI-1 protein levels and DNA binding respectively (Ouchida *et al*, 1995; Kovar *et al*, 1996; Tanaka *et al*, 1997; Silvany *et al*, 2000). However, these approaches required high expression levels to affect tumour cell growth. In this study, we have tested whether targeting a strong transcriptional repressor to EWS/FLI-1 DNA binding sites would demonstrate more efficient suppression of EWS/FLI-1 transformation. Thus, a specific repressor KF, which fused KRAB, a potent repression module, to the FLI-1 DNA binding domain was transfected into EWS/FLI-1 transformed cells and a human PNET cell line. All *EWS/FLI-1* transfected cell lines and the human PNET cell line, SK-N-MC, showed inhibition of proliferation rate in low serum media, loss anchorage-independent ability in soft agar assay and impairment of tumour formation in *BALB/c nu/nu* mice. This is a new approach to inhibit the transformation phenotypes of PNET using a specific repressor without removal of the *EWS/FLI-1* oncogene.

Although the mechanism of the KRAB fusion protein inhibition requires further investigation, KRAB heterologous repressors have been successful in interfering with endogenous cellular activators and even reversing malignant growth (Beerli *et al*, 1998; Ma *et al*, 1999; Fredericks *et al*, 2000). The KRAB domain has been shown to repress when placed at the amino or carboxy-terminus of a DNA-binding domain (Margolin *et al*, 1994). Thus, the KRAB module was fused to the *FLI-1* DNA binding domain to generate a chimaeric protein of similar structure to EWS/FLI-1 fusion protein. This KF is proposed not only to compete with EWS/FLI-1 for the ETS binding sites of downstream target genes but also repress the transcription activity by either directly inhibiting the transcriptional machinery and/or by altering the chromatin structure (Le Douarin *et al*, 1996; Moosmann *et al*, 1996).

In Chapter 3, both murine and human EWS/FLI-1 have been shown to have similar oncogenic potential and both fusion proteins appear to dysregulate similar downstream target genes and activate similar oncogenic pathways. Thus both murine and human EWS/FLI-1 transformed cell lines were used to study the effect of KF on reversion of

ES/PNET malignant phenotype. The *mutant KF* fusion gene was used as a negative control because it lacks the KRAB suppressive ability though it contains the same FLI-1 EDB domain. The KF was also transfected into normal NIH3T3 cells and the results (data not shown) indicated that there was no significant non-specific down-regulation on normal expression levels of genes which may change the normal cell cycle. This analysis demonstrated that the KF repressor could efficiently inhibit the tumourigenic phenotype in both murine and human EWS/FLI-1 transformed NIH3T3. In contrast, mutant KF could not suppress even though the mutation does not affect DNA binding. This showed that the reversion of EWS/FLI-1 transformed phenotypes in murine and human EWS/FLI-1 transformed cell lines was due to active repression from the KRAB domain in KF repressor. In addition, there was no change in normal cell cycle or the expression of other genes such as c-myc in normal fibroblasts expressing KF, indicating that the KF does not affect transcriptional activity in normal cells. On the other hand, the genes overexpressed in EWS/FLI-1 transformation of NIH3T3 cells were repressed by KF.

In this study, the expression levels of some selected downstream genes such as *stromelysin-1*, *manic fringe*, *EAT-2* and *c-myc* were down-regulated in KF co-transfectants. These genes such as *c-myc*, *stromelysin-1*, *Manic Fringe*, *mE2C* and *EAT-2* etc. are upregulated in EWS/FLI-1 transformed NIH3T3 cells (Bailly *et al*, 1994; Braun *et al*, 1995; Thompson *et al*, 1996; May *et al*, 1997; Arvand *et al*, 1998). This down-regulation also correlated with inhibition of the transformed phenotypes.

In particular, c-myc was activated in murine and human EWS/FLI-1 but was repressed by KF repressor. The *c-myc* gene has a critical role in the control of cellular proliferation and over-expression of c-myc proteins stimulates cell cycle progression, causes transformation, blocks differentiation, induces apoptosis in low serum (Henriksson *et al*, 1996), and is associated with a variety of tumours including ES/PNET (Bailly *et al*, 1994). Thus alteration in the regulation of c-myc might explain why the proliferation rate was reduced, the anchorage-independent growth potential was inhibited and the tumour development in nude mice was impaired in those EWS/FLI-1 transformed cells when KF repressor expressed.

SK-N-MC cells have a type I EWS/FLI-1 fusion. However, this cell line has other defects including the absence of p53 and proliferates rapidly, potentially due to the high expression of EWS/FLI-1 observed in these cells (Tanaka *et al*, 1996). The data in this study demonstrates that transfection of KF into these cells alters the proliferation rate, cell cluster morphology in low serum media and tumour formation ability in nude mice. This suppression effect was not as pronounced as that in EWS/FLI-1 transformed NIH3T3 cells. This was supported by the levels of mRNA and protein of c-myc in KF transfectants. Transfectants of the ES/PNET cell line SK-N-MC, which expressed KF at a much lower level than EWS/FLI-1, had only a subtle decrease in the levels of c-myc and less inhibition of the transformed phenotypes compared with EWS/FLI-1 transformed fibroblasts. A clone with a relatively high expression of KF however had a greater reduction in levels of *c-myc*. Thus, the level of *c-myc* appears to correlate both with the relative ratio of EWS/FLI-1 to KF and the efficiency of transformation. The less efficient inhibition of SK-N-MC transformation and c-myc activation by KF may also reflect the effects of other mutations in these cells. It is well known that the human cancer is caused by multiple mutation events and there is evidence that *c-myc* is not a direct target of EWS/FLI-1 (Bailly *et al*, 1994). However, KF still can efficiently suppress the malignant phenotype of SK-N-MC. This indicates there are other primary targets are also important in ES/PNET tumourigenesis and they might be suppressed by KF. The cell models developed in this study will be a good tool to discover these putative primary targets.

Since c-myc appears to be indirectly upregulated by EWS/FLI-1 and down-regulated by KF, there must be some intermediate factors which contribute to transformation. It is likely that EWS/FLI-1 directly interacts with a small number of target genes whose products subsequently modulate the activities of other genes. These cell lines will provide excellent reagents to identify these primary target genes combined with micro-array analysis. Identifying such primary target genes will be a crucial step in determining the biochemical mechanism of action of EWS/FLI-1.

Stromelysin-1 is one potential primary target gene which has been demonstrated to be up-regulated by EWS/FLI-1 (Braun *et al*, 1995) and has two tandem ETS binding motifs are present in the *stromelysin-1* promoter that are responsive to ETS-1, ETS-2 and TEL (Wasylyk *et al*, 1991, 1992; Fenrick *et al*, 2000). In Chapter 3, the

stromelysin-1 gene was shown to be up-regulated in the EWS/FLI-1 transfected cells by Western blot and semi-quantitative RT-PCR. Furthermore, transient transfection of St-Luc reporter vector in mEF, HuEF transformed cells and SK-N-MC also showed increased activity of luciferase, indicating EWS/FLI-1 may upregulate of *stromelysin-1*. However, expression was not altered after mutation of an ETS binding site in this 1.4 kb promoter. Besides, the expression was also not changed in KF containing cells and did not correlate with gene expression. These data indicate that the ETS binding sites for EWS/FLI-1 and KF are not presenting in this 1.4 kb mouse *stromelysin-1* promoter or perhaps there are other factors. Recent reports suggest that the oncogenic role of EWS/FLI-1 in activation of target genes may not strictly depend upon the DNA binding domain but may also be mediated by protein-protein interaction (Lin *et al*, 1999; Jaishankar *et al*, 1999; Olsen *et al*, 2001; Rossow *et al*, 2001; Zhou *et al*, 2001). This can be further explained why there was no complete inhibition of EWS/FLI-1 transformed phenotype by KF in this study. Thus, *stromelysin-1* may be an indirect downstream target gene of EWS/FLI-1 but further investigation such as using longer promoter of *stromelysin-1* in the similar assay is necessary.

In summary, the data showed in this Chapter demonstrate that KF can reverse the malignant phenotype of EWS/FLI-1 transformed NIH3T3 cells and a human PNET cell line SK-N-MC by repressing EWS/FLI-1 downstream target genes such as *c-myc*, *stromelysin-1*, *manic fringe* and *EAT2* genes. In addition, *c-myc* mRNA and protein levels appeared to correlate with the EWS/FLI-1 : KF ratio and the transformed phenotype. This indicates that KF can interfere with signaling pathways in transformation induced by EWS/FLI-1. Moreover, this study provides an approach to elucidate the mechanism by which the EWS/FLI-1 fusion gene regulates these downstream targets and identify the primary target genes of EWS/FLI-1 which will assist in the development of new therapeutic strategies.

Chapter 5

A Novel *ETS* Gene, *ER99*, is Upregulated by EWS/FLI-1 and is Expressed in ES/PNET

5.1 Introduction

The transformation in ES/PNET is believed to be mainly due to the dysregulation of FLI-1 downstream targets by EWS/FLI-1. Identification such downstream target genes will be a crucial step in determining the biochemical mechanism of action of EWS/FLI-1 and will assist the development of clinical therapy for ES/PNET. In our laboratory, a novel *ETS* gene was identified. The expression analysis showed that this gene is normally detected only in the normal human testis. However, it was shown to be expressed in most human cancer cells tested including ES/PNET. Further, it was also upregulated by EWS/FLI-1 in NIH3T3 cells. This indicates that there may be an important role for this ETS member in cancer development.

There have recently been an increasing reports of ETS transcription factors associated with transformation and oncogenesis. In these reports, the ETS transcription factors displayed abnormal expression due to induction or activation by virus, chromosomal translocations or rearrangement. For example, an acute avian leukemia virus, E26, induced a nuclear fusion protein, vMyb-vEts, in mixed erythroid and myeloid leukaemia in avian species (Metz *et al*, 1991a). Abnormally activated FLI-1 gene occurs after insertion of the Friend murine leukaemia virus (F-MuLV) into the FLI-1 locus in erythroleukaemias (Ben-David *et al*, 1991). The Spi-1 (PU.1) locus is rearranged in erythroid tumours caused by FV-P and FV-A complexes of spleen focus-forming virus (SFFV-P and –A) and F-MuLV (Moreau-Gachelin *et al*, 1988, 1989). Moreover, chromosomal translocations which rearrange the *ETS* genes are frequently associated with human sarcomas and leukaemias. For example, almost all ES/PNET are associated

with chromosomal translocations which fuses the N-terminus of EWS to the EDB domains of FLI-1, ERG, ETV-1, E1AF or FEV (Delattre *et al*, 1992; Sorensen *et al*, 1994; Jeon *et al*, 1995; Kaneko *et al*, 1996; Peter *et al*, 1997). The N-terminal domain of a EWS-related protein TLS/FUS fuses the ETS domain of ETV6/TEL or Erg-2 in certain types of human leukaemia such as chronic myeloid leukaemia (CML) (Ichikawa *et al*, 1994; Panagopoulos *et al*, 1994). This *TEL* gene is also fused by *PDGFβ* and *AML1* gene fuse in chronic myelomonocytic leukaemias and acute lymphoblastic leukaemia respectively (Golub *et al*, 1994, 1995). All of the above tumours are due to the abnormal expression of ETS genes by the viral inductions and chromosomal translocation. All these events retain the functional EDB domain of ETS genes and the fusion proteins gain the abnormal high transactivation activity. Thus, the tumourigenesis in these tumours may be due to the dysregulations of the ETS downstream target genes.

The overexpression of *ETS* genes also occurs in many human cancers. For example, ETS-1 is amplified in myelodisplastic syndrome (Ohyashiki *et al*, 1990), breast carcinoma (Delannoy-Courdent A, *et al*, 1998) and endometrical carcinoma (Takai *et al*, 2000). High expression of ETS-2 may increase susceptibility to leukaemia in Down's syndrome (Sacchi *et al*, 1988) and an acute nonlymphoblastic leukaemia (Santoro *et al*, 1992). Furthermore, some ETS proteins including PEA3, ER81, ERM and Elf3/ESX/ESE-1 have also been found to be overexpressed in mammary epithelial carcinoma cells, (Monte *et al*, 1995; Chen *et al*, 1996; Tymms *et al*, 1997; Chang *et al*, 1997; Oettgen *et al*, 1997). These reports indicate the high expression of ETS transcription factors may play an oncogenic role in tumourigenesis of human carcinomas.

Due to the increasing evidence for the involvement of ETS factors in human tumourigenesis, our laboratory has explored whether other novel *ETS* genes may be involved in the development of human cancers. Thus, in this study, novel *ETS* genes were isolated from a spectrum of *ETS* genes expressed in a human breast cancer cell line using a degenerative PCR approach to amplify the ETS binding domain region of ETS transcription factors. In this study, a novel *ETS* gene, designated *ER99*, was isolated and found to be expressed in ES/PNET cell lines. This novel gene was

regulated by EWS/FLI-1, suggesting that it may be involved in the aetiology of ES/PNET. This Chapter describes the isolation of this novel *ETS* gene and its expression patterns in human normal tissues and numerous human cancer cell lines including ES/PNET.

5.2 Results:

5.2-1 Isolation of *ER99*

To isolate novel *ETS* genes from human cancers, a pair of degenerative primers was designed to amplify a spectrum of *ETS* genes expressed in a human breast cancer cell line, ZR-75-1. Pure Poly(A)$^+$ RNA was isolated from this cell line using the methods described in Sections 2.4-1 and 2.4-2. The first strand cDNA was synthesized by reverse transcriptase kit (Promega) according to manufacturer's instructions. *Taq* polymerase PCR was performed using a pair of degenerative primers: degS and degA (oligonucleotide sequences see Appendix 4) (Aryee *et al*, 1998) with 30 cycles of denaturation (94^{0}C for 30 seconds), annealing (55^{0}C for 30 seconds) and extension (72^{0}C for 1 minute), preceded by a primary denaturation step (94^{0}C for 5 minutes) and followed by a final extension step (72^{0}C for 5 minutes) after the last cycle carried in a thermal cycler (Perkin Elmer). The primers designed according to the conserved sequences of the EDB domain. The PCR fragments with ~245 bp size were pointed and excised from agarose gel and purified by 'GeneClean' (see Section 2.2-3) (Fig.5.1). The purified PCR fragments were digested by *Hind*III and *Eco*RI as the degenerative primers contain these sites were subcloned into the same sites of pBluescript KS+ vector (Fig.5.1). Eleven positive clones were identified by using *Hind*III/*Eco*RI digestion screening and PCR using T7 and T3 primers on the plasmid DNA prepared from mini-preparation of plasmids (Fig.5.1). After sequencing, five clones were identified as *ETS-2*, 3 clones were *ELFR* and 2 clones were *ER71* like gene (designated to be *ER99* in this study) (Fig.5.2A).

5.2-2 Amplification 3' Region of *ER99* by 3' RACE PCR

In order to amplify the 3' region from EDB domain to poly (A)$^+$ of *ER99*, a sense primer (3S 5'-CTCTCACCGTTTGCTCCGAACCGAGC-3') and an anti-sense primer Oligo dT were used in *Taq* polymerase PCR on the first strand cDNA of ZR-75-1 (Fig.5.2B). A 400 bp PCR fragment was amplified in this PCR and identified on agarose gel. This fragment was excised, purified and sub-cloned into pGEMT vector for

Degenerative PCR

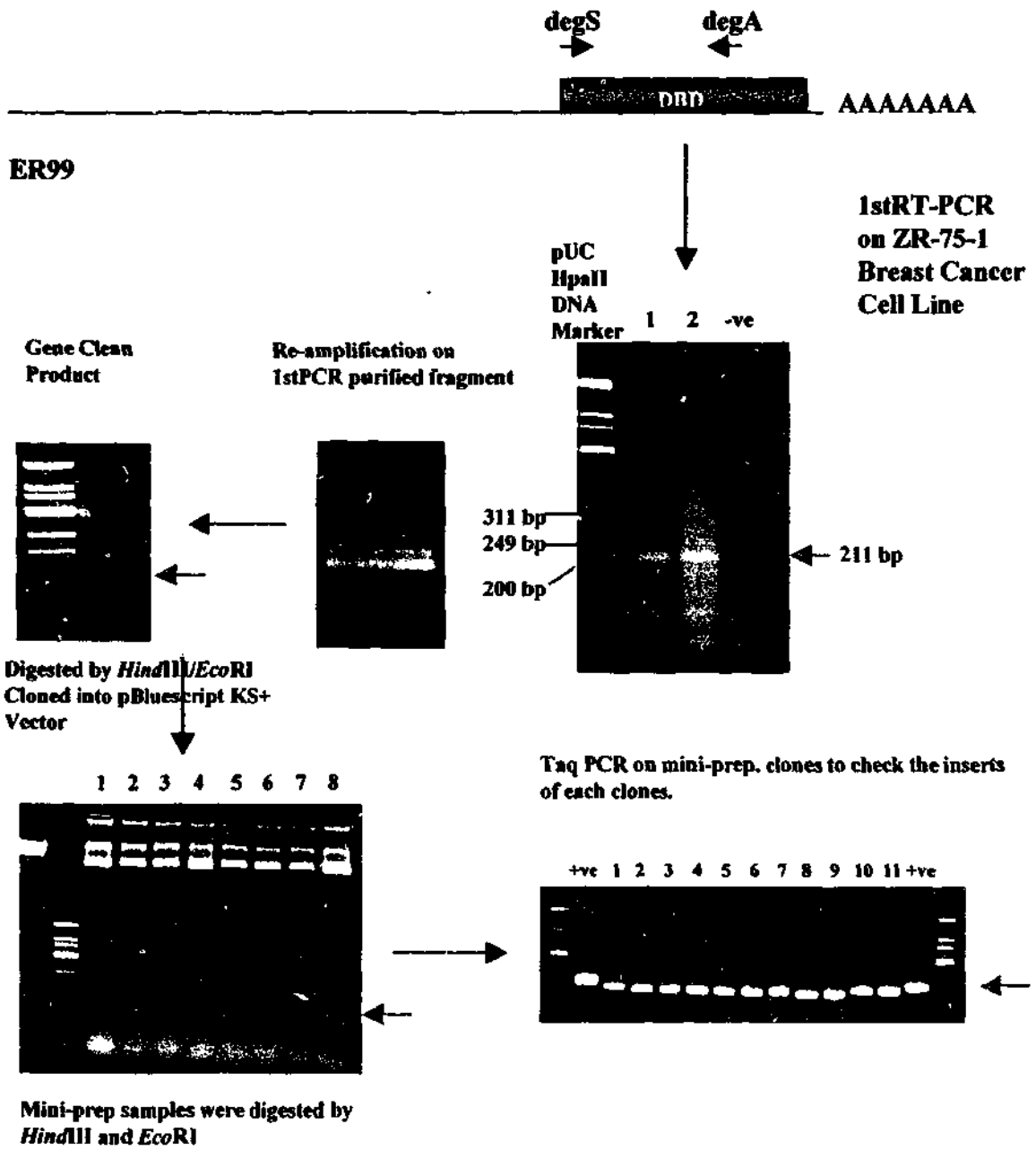
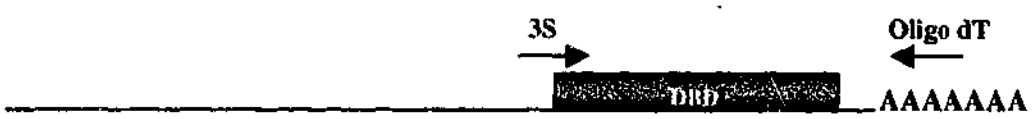


Fig.5.1 Schematic diagram and photos showing the cloning of a novel *ETS* gene, *ER99*, by degenerative PCR from the ZR-75-1 breast cancer cell line. The PCR amplified a ~ 211 bp band which was isolated and sub-cloned into pBluescript KS+ vector at *Hind*III and *Eco*RI sites.

A

```
CACCGTTTGCTCCGAACCGAGCCCGCAGTCGGACCGTGCCAGTTTGGCTCGATGCCCCAAAACTAACCACCGAGGTCCCATTCAGCTGTG  90
GCAGTTCCTCCTGGAGCTGCTCCACGACGGGGCGCGTAGCAGCTGCATCCGTTGGACTGCCGTGGCTCGGCTGTGGGGCGAGCGCAAGAG 180
AAAGCCGGGCATGAATTACGAGAAGCTGAG                                                             211
```

B **3' RACE PCR**



C

```
                                                                                  G  P  I  Q  L
TCTCACCGTTTGCTCCGAACCGAGCCCGCAGTCGGACCGTGCCAGTTTGGCTCGATGCCCCAAAACTAACCACCGAGGTCCCATTCAGCT  90
 W  Q  F  L  L  E  L  L  H  D  G  A  R  S  S  C  I  R  W  T  G  N  S  R  E  F  Q  L  C  D
GTGGCAGTTCCTCCTGGAGCTGCTCCACGACGGGGCGCGTAGCAGCTGCATCCGTTGGACTGGCAACAGCCGCGAGTTCCAGCTGTGCGA 180
 P  K  E  V  A  R  L  W  G  E  R  K  R  K  P  G  M  N  Y  E  K  L
CCCCAAAGAGGTGGCTCGGCTGTGGGGCGAGCGCAAGAGAAAGCCGGGATGAATTACGAGAAGCTGAGCCGGGGCCTTCGCTACTACTAC 270

GCCGCGACATCGTGCGCAAGAGCGGGGGGCGAAAGTACACGTACCGCTTCGGGGGCCGCGTGCCCAGCCTAGCCTATCCGGACTGTGCGG 330

GAGGCGGACGGGGAGCAGAGACACAATAAAAATTCCCGGTCAAACCAAAAAAAAAAAAAAAA                             391
```

D **Human Cosmids Ch. 19q13.1 (1mb)**

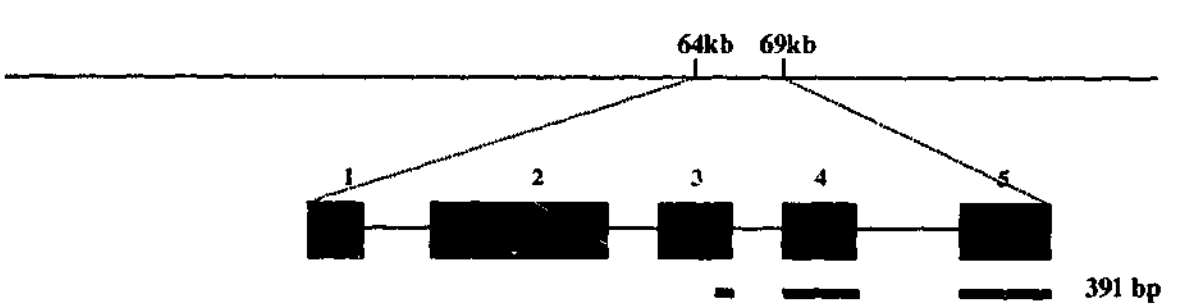


Fig.5.2 The nucleotide sequence of 3' region of *ER99* partial cDNA (A) The nucleotide sequence of partial *ER99* cDNA isolated from degenerative PCR. (B) The schematic diagrams showing on isolation of 3' region of *ER99* by 3' RACE PCR. (C) The nucleotide sequence of 3' region of *ER99*. The deduced amino acid sequences showing the EDB domain. (D) The predicted genomic structure and localization of *ER99* at human cosmids Ch. 19q13.1. The exons are shown to scale as solid boxes. The partial cDNA and their exons of the partial isolated *ER99* cDNA are shown in lines.

sequencing. The sequence data showed that this fragment contains an *TAG* stop codon, *AATAAAAA* stop signal and poly A signal sequences behind the EDB domain of this ER99 (Fig.5.2C).

5.2-3 Amplification of 5' Region of *ER99* by 5' RACE PCR

To amplify the 5' region of ER99, the 5' RACE System for Rapid Amplification of cDNA Ends was used on ZR-75-1 cell line (GIBCO BRL). The GSP1 (GST 5'-CGATAGTAGTAGCGAAGGCCCC-3') was used for synthesizing the first strand cDNA of ZR-75-1. Following cDNA synthesis, the first strand product was purified from unincorporated dNTPs and GSP1. The terminal deoxynucleotidyl transferase (TdT) was used to add dCTP at the 5' end of cDNA. An anti-sense primer (2A-RACE 5'-GCTCAGCTTCTCGTAATTCATGCC-3') and the commercial sense primer, 5' RACE Abridged Anchor Primer was then amplified on this cDNA. Lastly, a nested PCR was used on this PCR product by another inner anti-sense primer (3A 5'-CCGACTGCGGGCTCGGTTCGGAGCAAACGGTG-3') with another commercial primer, UAUP. Several PCR fragments were generated with sizes ranging from 500 bp to 700 bp which were subcloned into pGEMT vector (data not shown). Sequence analysis found that these gene fragments were other known genes according the BLAST searching results (data not shown). Thus another higher specific anti-sense primers were designed and 5'RACE PCR will be continued as further studies.

5.2-4 Characterization of *ER99*

From the sequence of 3' region of *ER99*, the sequence similarity of *ER99* and other genes was searched through both BLAST software of NCBI (www.ncbi.nlm.nih.org) and Celera Discovery System of Celera Co. (www.celera.com). The search results showed that the sequence of *ER99* was highly similar to murine *ER71* and genomic sequence of Human DNA from overlapping chromosome 19 cosmids R31396, F25451 (Accession number: AC002115), Homo sapiens chromosome 19 working draft sequence segment (Accession number: NT_011296, NCBI Human Genome Project)

(GA x2KMHMQW42M: 500001..1000000, Celera Discovery System). All the data shows that this gene localizes at human chromosome 19q13.1. From the genomic sequence of Homo sapiens chromosome 19 working draft sequence segment, the 3' partial cDNA of *ER99* is identical to 3 regions on this segments; 1-79 bp of *ER99* (Fig.5.2C) 100% identical to segments 35928200-35928270, 76-194 bp of *ER99* (Fig.5.2C) 100% identical to segments 35927600-35927720, and 189-406 bp of *ER99* (Fig.5.2C) 99% identical to segments 35927080-35927300. The orientation of this gene is from centromere to telomere according the map of human chromosome 19 (NCBI and Celera Human Genome Projects). There are no known or predicted genes in the same locus (data from Celera Discovery System). However, form the sequence of Human DNA from overlapping chromosome 19 cosmids R31396, F25451 (Accession number: AC002115 and the software of GENESCAN 1.0, the cDNA of *ER99* was predicted to have 5 exons reading-frame with 1086 bp, encodes 361 amino acids with 36.6 kDa. The 3' region of *ER99* obtained in this study consists of 3 exons separated by two introns which were 304 bp and 481 bp (Fig.5.2D).

By comparing murine *ER71* and the predicted cDNA of *ER99*, both EDB domains share 98.2% identity in amino acid sequence (Fig.5.3). The adjacent protein sequences at both sides of EDB domain are also share high identity with 75% to 91.7% (Fig.5.3). Additionally, the region near initiation codon of the predicted cDNA and murine ER71 also share ~87% identity (Fig.5.3). However, at the middle region from 32 a.a. to 176 a.a. of the predicted cDNA, *ER99* shared only 20% identity to murine *ER71*(Fig.5.3). The overall identity between predicted *ER99* and murine *ER71* is 60.3% according to the analysis software of PCGENE. Thus, *ER99* may not the human orthologue of murine *ER71*, although the human *ER71* has not been isolated. It may be the novel gene in *ETS* transcription family but shares high sequence similarity in ETS binding domain of murine *ER71*. To investigate this further, a sense primer near the putative transcription initiation point with high nucleotide sequence identity between murine *ER71* and human *ER99* and one anti-sense primer at EDB domain was used in RT-PCR on the ZR-75-1 cDNA library. However, this RT-PCR did not generate any PCR product which may indicate that this human *ER99* was not the human *ER71* gene (data not shown).

```
MUER71    -          MDLWNWDEASLQEVPPGDKLTGLGAEFGFYFPEVALQEDTP -41
ER99      - NIQKAFIASMDLWNWDEASPQEVPPGNKLAGL--E-----P------DS- -36

MUER71    - ITPMNVEGCWKGFPELDWNPALPHEDVPFQAEPVAHPLPWSRDWTDLGCN -91
ER99      - ----------------------------QA------LPWSGDWTDMAC- -50

MUER71    - TSD-PWSCASQTPGPAPPGTSPSPFV--GFEGATGQNP--A SAGGVPSW -136
ER99      - TAWDSWSGASQTLGPAPLG--PGPIPAAGSEGAAGQNCVPV--AGEATSW -96

MUER71    - SHPPAAWSTTSWDCSVGPSGATYWDNGLGGEAHEDYKMS----WGGSAGS -182
ER99      - SRAQAAGSNTSWDCSVGPDGDTYWGSGLGGEP----RTDCTISWGGPAGP -142

MUER71    - DYTTTWNTGLQDCSIPFEGHQ----------SPAFTT--P-SK-SNKQSD -218
ER99      - DCTTSWNPGL---------HAGGTTSLKRYQSSALTVCSEPSP----QSD -179

MUER71    - RATLTRYSKTNHR                                      -268
ER99      - RASLARCPKTNHR                                      -229

MUER71    -                        SRGLRYYYRRDIVL-KSGGRKYTYRFGGRV -317
ER99      -                        SRGLRYYYRRDIVR-KSGGRKYTYRFGGRV -278

MUER71    - PV-LAYQDD-----------M---G-------H---L----P------- -330
ER99      - PS-LAYPDCPASRLRLHSLRNLRGPPSSVVSFGMRNHPPPVLRNDQRRPR -327

MUER71    - ----GAE-----G-Q                          -335

ER99      - LREPAMCPPCDERGKETKPKAHARAYCVPALCRDL -362
```

Fig.5.3 The alignment of the predicted amino acid sequences of *ER99* with murine *ER71*. The blue shaded box represents the ETS binding domain.

5.2-5 Expression of *ER99* in Normal Human Tissues

The size and expression pattern of *ER99* were determined by Northern blot analysis in a wide variety of tissues on the commercial human normal tissues blot (Clontech). By using a 211 bp *ER99* (EDB domain of *ER99*) as a probe, a ~1.4kb *ER99* transcript was identified in human testis tissue only (Fig.5.4). There was no other extra bands in addition to this 1.4 kb transcript (Fig.5.4), indicating that this *ER99* is restrictedly expressed in human testis tissue and without alternative splicing. The 1.4 kb size transcript was consistent with the predicted cDNA (1086 bp plus the 5' and 3' untranslated regions).

5.2-6 Expression of *ER99* in Variety of Human Cancer Cell Lines

Since *ER99* was isolated from ZR-75-1 human breast cancer cell line and expression was restricted to normal human testis, it was interesting to investigate the expression pattern in other human cancer cell lines. GeneBank of NCBI, indicates that two groups have also identified a partial cDNA of this gene. Dr. R Strausberg from National Cancer Institute found this gene from human gastric tumour (unpublished data from GeneBank accession number: AW170266), and Drs. DNT Aryee and H Kovar from Children's Cancer Research Institute, St. Anna Kinderspital, Austria, also found this gene co-expressed with EWS/FLI-1 in Ewing's sarcoma cell line (unpublished data from GeneBank accession number: AF000671). Thus the presence of this gene in other human or murine cancer cell lines was examined in this study. Two specific primers, ER99-3S and ER99-2A (Appendix 4), were used in RT-PCR to amplify a 211bp fragment which demonstrated the expression of *ER99* in mouse and human normal and cancer cell lines. Results found that neither any normal murine mammary cell line nor 2 murine breast cancer cell lines had *ER99* expression (Fig.5.5). On the other hand, in all human cancer cell lines, except MDA-MB-453, including the pancreatic tumour cell line, and four human breast cancer cell lines ZR-75-1, T-47D, MCF-P7 and MDA-MB-435 had *ER99* expression (Fig.5.5). Among these *ER99* expressed cell lines, ZR-75-1 had highest *ER99* expression level (Fig.5.5). These results indicate that there is a *ETS* 'cancer-testis' gene expressed during cancer development.

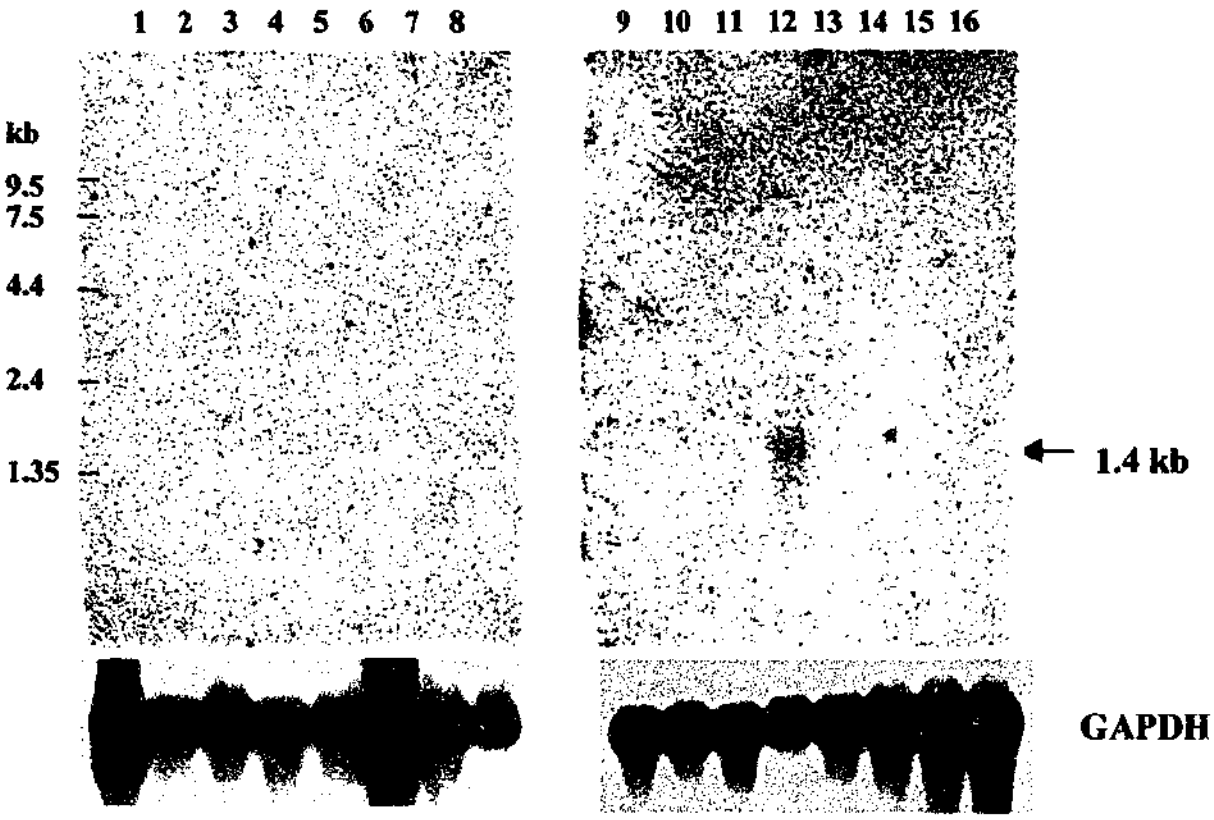


Fig.5.4 The size and expression pattern of *ER99* showed by Northern blot analysis on human normal tissues. The 211 bp of *ER99* (ETS binding domain) was used as a probe on the commercial normal human tissue northern blots (Clontech). Lanes 1 to 16 represent (1) heart, (2) brain, (3) placenta, (4) lung, (5) liver, (6) skeleton muscle, (7) kidney, (8) pancreas, (9) spleen, (10) thymus, (11) prostate, (12) testis, (13) ovary, (14) small intestine, (15) colon (mucosal lining) and (16)peripheral blood leukocyte.

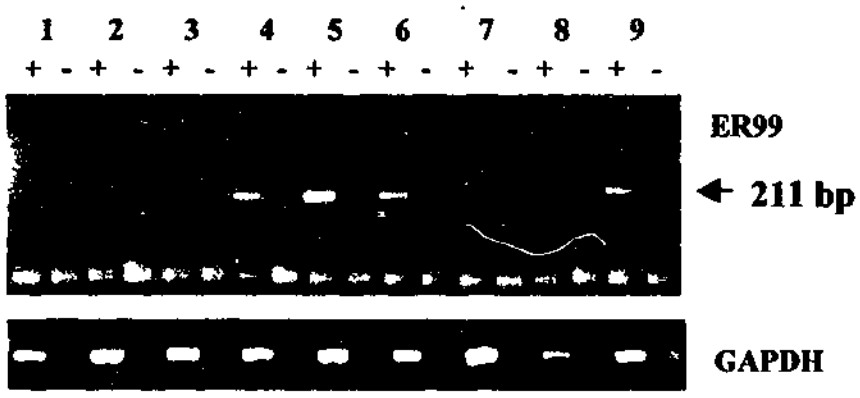
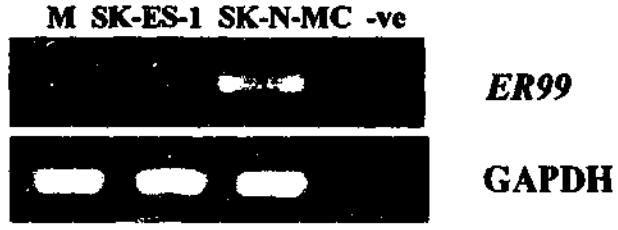


Fig.5.5 The expression of *ER99* in numerous murine and human cancer cell lines as detected by RT-PCR. (1) Mouse mammary cell line. (2) BMB1 murine breast cancer cell line. (3) BML1 murine breast cancer cell line. (4) Human pancreatic tumour cell line. (5) ZR-75-1 human breast cancer cell line. (6) T-47D human breast cancer cell line. (7) MCF-P7 human breast cancer cell line. (8) MDA-MB-453 human breast cancer cell line. (9) MDA-MB-435 human breast cancer cell line.

A) The Expression of *ER99* in ES/PNET Cell Lines



B) The Upregulation of *ER99* in Human and Mouse EWS/FLI-1 Transformed Cell Lines

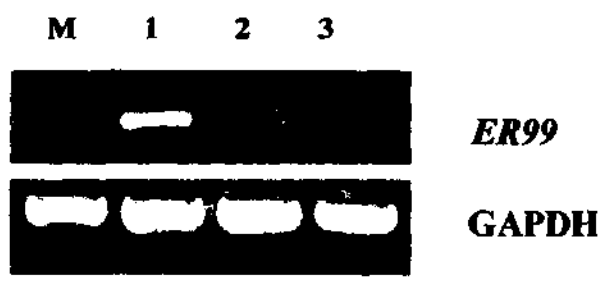


Fig.5.6 The expression of *ER99* in human ES/PNET and EWS/FLI-1 transformed cell lines,shown by RT-PCR. (A) (M) NIH3T3 mock cells, Human ES/PNET cell lines; SK-ES-1 and SK-N-MC, and water control . (B) *ER99* is upregulated in human and mouse EWS/FLI-1 transformed NIH3T3 cell lines (1) Human EWS/FLI-1 transformed cell line HuEF#16, (2) murine EWS/Fli-1 transfomred cell line mEF#8, and (3) mEF#1.

5.2-7 *ER99* is Expressed in *EWS/FLI-1* Expressing Cell Lines

In this study, the expression of this gene was detected in three human ES/PNET cell lines including SK-ES-1 and SK-N-MC by RT-PCR (Fig.5.6 A). Furthermore, this gene was also shown to be expressed in both human and mouse EWS/FLI-1 transformed NIH3T3 cells but not in NIH3T3 normal cells (Fig.5.6B). This indicated that this gene might be up-regulated by EWS/FLI-1 in NIH3T3 cells.

5.3 Discussion

By using degenerative PCR, a human novel *ETS* gene, designated *ER99*, was identified in this study. This gene is expressed only in normal human testis but was abnormally expressed in numerous cancer cells and upregulated by EWS/FLI-1.

In this study, the 5' region of *ER99* hasn't been obtained. However, the 3' region of *ER99* showed that this gene is located at human chromosome 19q13.1 according the gene search in both BLAST software of NCBI (www.ncbi.nlm.nih.org) and Celera Discovery System of Celera Co. (www.celera.com). Although the EDB domain of ER99 was 98.2% identity in amino acid sequence to murine ER71, this human ER99 has been shown to be a novel ETS member due to the difference of predicted amino acid sequence of ER99 and murine ER71. These two proteins share high identity only at ETS binding domain, small parts adjacent ETS binding domain and at the initiation point. There was no homology for the other regions. This shows that *ER99* is another ETS member. To further identify this novel gene is not the human *ER71* gene, specific primers correlate to the identical regions between of murine *ER71* and *ER99* were designed and used for RT-PCR on *ER99* positive cell lines. Results found that there are no amplified fragments, indicating that the *ER99* is not the human *ER71* gene and may be another novel ETS member.

From Northern blot analysis on a variety of normal human tissues, the expression of *ER99* was observed only in the testis. This transcript was a ~1.4kb mRNA with no evidence of alternative spliced transcripts. Sequence analysis of 3' region cDNA of this gene demonstrated that the 3' untranslated region was small. In addition, this is the first report of a human *ETS* gene restrictedly expressed in testis. Thus ER99 may regulate the transcriptional activities in testis specific processes such as spermatogenesis or perhaps in the control of proliferation or germ cell division. However, these cells expressing this gene and its function require further investigation by *in situ* hybridization or gene knockout strategy.

A significant finding in this study was the expression of this gene in numerous human cancer cell lines including human breast cancer cell lines and a pancreatic tumour cell

line. These data correlated with other unpublished reports from Dr. R Strausberg (ref: GeneBank accession number: AW170266) and Drs. DNT Aryee and H Kovar (ref: GeneBank accession number: AF000671) who also found that this gene is expressed in human gastric tumour and Ewing's sarcoma cell lines. All these cancers originate from epithelial or neroectodermal cells. However, *ER99* mRNA was not observed in tissues containing these cells by Northern blot. Thus *ER99* appears to be switched on during tumourigenesis of these cancers and indicates that this testis *ETS* gene is 'cancer-testis' gene. There are many reports shown that these 'cancer-testis' genes are involved in a wide range of human tumours such as *MAGE-3, NY-ESO-1, HOM-MEL-4/SSX-2, SSX-1, SSX-4, HOM-TES-14/SCP-1* in human brain tumour (Sahin *et al*, 2000), CT9 expresses in lung cancer (Scanlan *et al*, 2000), *WWOX* and *ING1* in breast cancer (Jager et al, 1999; Bednarek *et al*, 2000), *XAGE-1* in Ewing's sarcoma cell lines (Liu *et al*, 2000). Some of these 'cancer-testis' genes are associated with proliferation rate control such as the SNF-2 like gene (*PASG*) (Lee *et al*, 2000) and others are cell biochemical regulators such as the human serine protease testisin found in testicular abnormalities and tumours (Hooper *et al*, 2000). The restricted expression of these 'cancer-testis' proteins may indicate the potential use as targets for immunotherapy or gene therapy. Therefore, *ER99* may be a good candidate for the development of a novel gene therapy for cancers in the future.

In addition to *ER99* expression in Ewing's sarcoma cells, an intriguing finding in this study was that this gene could be upregulated by the *EWS/FLI-1* fusion gene. This indicates that there is some relationship between *ER99* and *EWS/FLI-1* genes. It will be interesting to clarify whether *ER99* is a primary target of EWS/FLI-1.

In summary, a novel *ETS* gene, *ER99*, was isolated by degenerative PCR. This gene encodes a 1.4 kb transcript and normally is only expressed in human testis tissue, however it is also expressed in numerous human cancers, suggesting that it is a 'testis-cancer' gene. The continued study on this gene will explore the novel gene therapy in ES/PNET and other human cancers.

Chapter 6

Characterization of Murine *EWS* Gene and Generation of *EWS* Gene Knockout and *EWS/Fli-1* Fusion Gene Knock-in Targeting Constructs

6.1 Introduction

EWS, TLS/FUS, hTAF$_{II}$68 and Cabeza all share a consensus RNA-binding domain (RNP-CS) and belong to a subfamily of RNP-CS-containing proteins called TET (Delattre *et al*, 1992; Crozat *et al*, 1993; Stolow *et al*, 1995; Bertolotti *et al*, 1996). This suggests that EWS may function as a RNA processing protein which is capable of binding RNA and /or ssDNA (Birnet *et al*, 1993; Burd *et al*, 1994). In addition, the region from amino acids 157 and 262 is homologous to the CTD of the large subunit of eukaryotic RNA polymerase II protein (CTD-pol II) suggesting that EWS might be involved in the Pol II transcription machinery (Corden, 1990; Bertolotti *et al*, 1996). Furthermore, EWS also contains an IQ domain that is phosphorylated by protein kinase (PKC) and interacts with calmodulin (CaM) (Deloulme *et al*, 1997). This IQ domain may function as a regulatory domain in EWS protein binding of RNA homopolymers (Deloulme *et al*, 1997).

The human *EWS* gene is ubiquitously expressed with two alternatively spliced transcripts, *EWS* and *EWS-b*, the shorter form lacking exons 8 and 9 (Ohino *et al*, 1994), and the human *EWS* gene promoter has a high content of unmethylated CpG islands (Bird, 1986; Plougastel *et al*, 1993). This is consistent with other genes necessary for normal cellular functions or "housekeeping genes", which are highly conserved through evolution (Bird, 1986; Plougastel *et al*, 1993). Similar to these "housekeeping genes". EWS may play an important role in normal cellular functions. Although the predicted

function of EWS has been verified *in vitro*, the analysis of an *EWS* gene knockout mouse would provide a better approach to elucidating its *in vivo* role.

The human *EWS* and closely related *TLS/FUS* genes have been shown to be involved of chromosomal translocations in numerous human tumours such as ES/PNET, Desmoplastic Small Round Cell Tumors (DSRCT), Malignant Melanoma of Soft Parts (MMSP), Myxoid Liposarcoma, Extraskeletal Myxoid Chrondrosarcoma (Delattre *et al*, 1992; Zucman *et al*, 1993; Crozat *et al*, 1993; Gerald *et al*, 1995; Clark *et al*, 1996). More than 10 different chimaeric structures of the fusion transcripts, due to the varying molecular breakpoints in the der(22), have been identified (May *et al*, 1993; Zucman *et al*, 1993; Delattre *et al*, 1994; Bhagirath *et al*, 1995). In Ewing's sarcoma, the breakpoints are located from introns 5 to 10 in the *EWS* gene. This suggests that there are many hot spots or fragile sites in some *EWS* introns which are favored for chromosomal translocations. Sequence analysis on these breakpoints revealed that the junctions were flanked by various oligomers such as 5'-AGAAAARDRR-3' (Obata *et al*, 1999), *Alu* repeats and/or eukaryotic topoisomerase II cleavage sites, 5'-RNYNNCNNGY$_{v}$NGKTNYNY-3' (Spitzner *et al*, 1989; Obata *et al*, 1999). A recent finding suggests that *Alu* richness in intron 6 of *EWS* could mediate the genome plasticity and the frequency of translocation in Ewing's sarcoma (Zucman *et al*, 1997).

This chapter describes the isolation of the murine *EWS* gene and comparison of mouse and human *EWS* expression and genomic structure. The introns 6 and 7 of murine *EWS*, which are the most frequent sites for translocations in Ewing's sarcoma, have been sequenced and analysed for similar motifs or repeat sequences found in human *EWS/FLI-1* fusion sites. In addition, mouse *EWS* pseudogenes were verified similar to that described for human *EWS*. Finally, this chapter describes the procedures of generating the knockout targeting vector for *EWS* gene at exon 4 which will enable the future generation of a *EWS* null mouse which will be important in the characterisation of EWS function *in vivo*. Furthermore, a "knock-in" construct has also been generated to couple the EDB domain of the *Fli-1* gene to exon 7 of *EWS*, mimicking the *EWS/FLI-1* translocation *in vivo*. This will enable the generation of a mouse model for Ewing's sarcoma.

6.2 Results:

6.2-1 Expression Pattern of *EWS* Gene in Human and Murine Tissues

Consistent with previous reports, both human and mouse *EWS* were ubiquitously expressed (Fig.6.1 and Fig.6.2). The human *EWS* mRNA two alternatively spliced transcripts, *EWS* and *EWS-b* with 2.5 and 2.6 kb in size respectively (Ohno *et al*, 1994; Aman *et al*, 1996)(Fig.6.1). The small difference in the band size of these two alternative transcripts may explain why they were not distinguished on this Northern blot. On the other hand, the 6.0 kb band is thought to be a pre-mRNA of *EWS* gene in all human tissues (Fig.6.1) (Ohno *et al*, 1994; Aman *et al*, 1996). The murine *EWS* transcript was larger than the human *EWS* transcript. The dominant transcript of murine *EWS* transcript is 3.0 kb and was expressed in all tissues and cell lines (Fig.6.2 and Fig.6.3). In this study, a 9.0 kb transcript was also co-expressed in all mouse tissues and cell lines (Fig.6.2 and Fig.6.3), which may be a pre-mRNA similar to that described for human (Ohno *et al*, 1994; Aman *et al*, 1996). Since the reading frame of both human and mouse *EWS* cDNAs are similar, the longer transcript of murine *EWS* mRNA may be due to increased 3' untranslated region and poly A^+ tail.

6.2-2 Pseudogenes of *EWS* in Mouse

To generate *EWS* knockout and *EWS/Fli-1* knock-in constructs, the *EWS* genomic fragments from exons 1 to 10 were required. The genomic fragments were screened from a 129SvJ gFIXII genomic library (Stratagene) using the human *EWS* partial cDNA (exons 1 to 7) as a probe. Screening identified 10 positive clones. However, sequence analysis on these clones revealed that they were pseudogenes of *EWS* gene. This was confirmed by using different combinations of primers recognized murine EWS exons 1 to 7. All the PCR results gave a fragment which was same size as wild-type murine *EWS* cDNA (data not shown). By sequence analysis, these pseudogenes revealed high nucleotide sequence identity to wild type *EWS* cDNA exons 1 to 7 without introns between each exons or polyA^+ tail at the 3'-end (Fig.6.4A and Fig.6.4B). In this region, about 40-60 mutations and 9-20 bp deletions were found when compared to the *EWS* cDNA sequence (Fig.6.4A and Fig.6.4B). The open reading frame of these pseudogenes

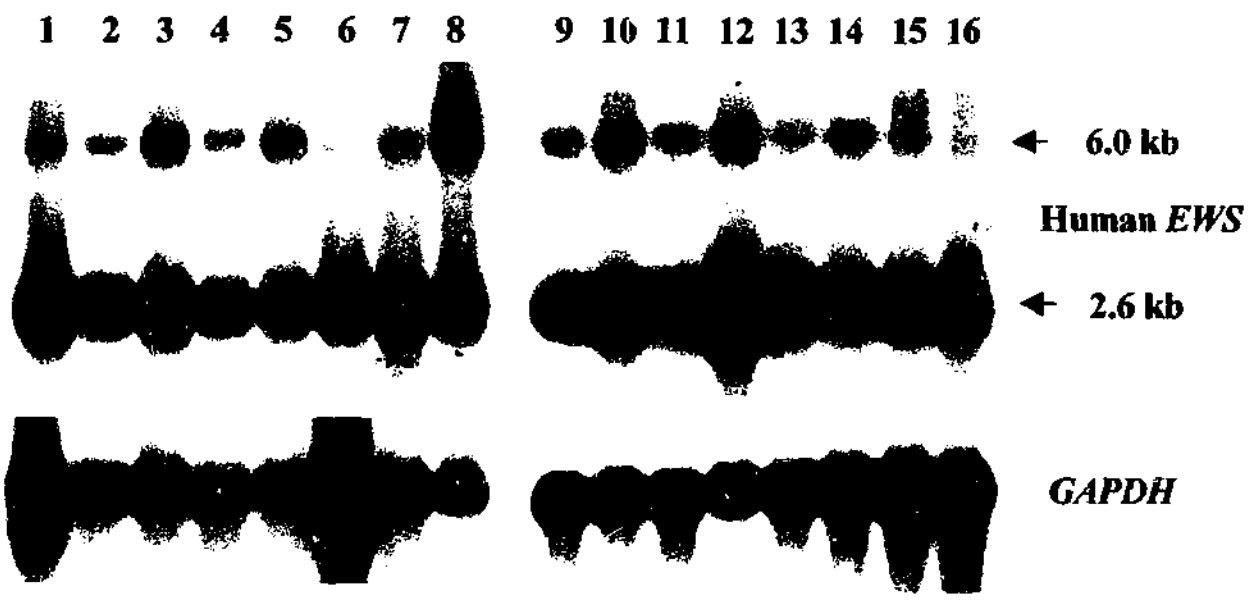


Fig.6.1 Northern analysis showing expression of human *EWS* gene in normal human tissues. Lanes 1 to 16 represent heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon (mucosal lining) and peripheral blood leukocyte respectively.

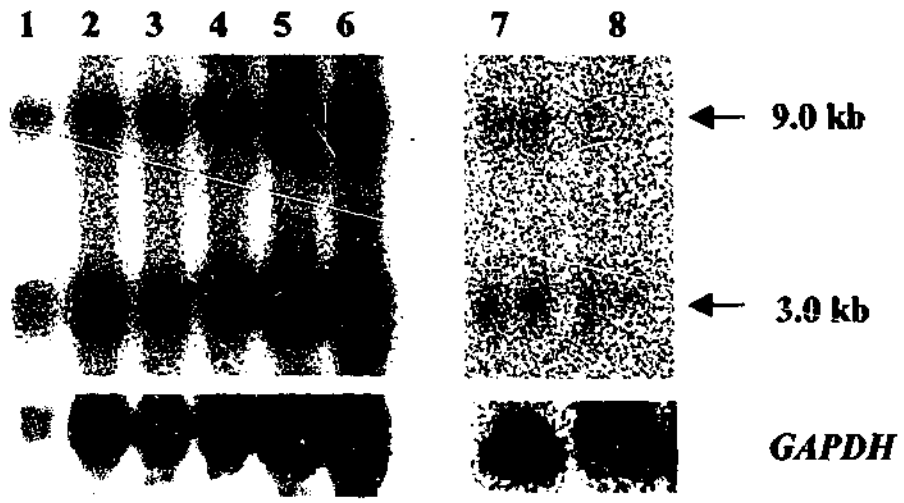


Fig.6.3 Northern analysis showing expression of murine *EWS* gene in mouse normal cell lines and tissues. Lanes 1 to 8 represent (1) mouse liver, (2) mouse salivary gland, (3) mouse skeletal muscle, (4) mouse large intestine, (5) mouse RAJ (T-cell) cell line, (6) mouse bone marrow, (7) mouse 129S6/SvErTac embryonic stem (ES) cell line and (8) mouse C57B1/6J embryonic stem (ES) cell line.

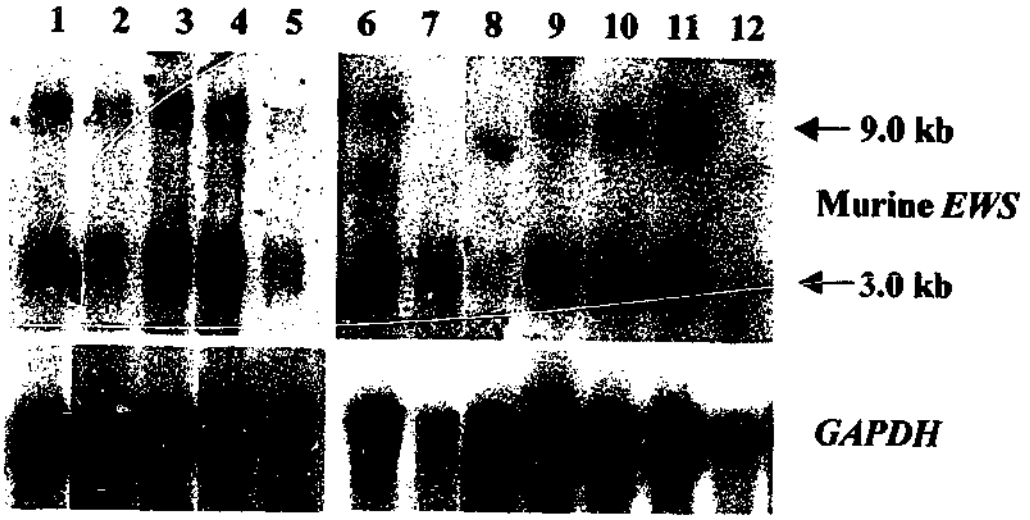


Fig.6.2 Northern analysis showing expression of murine *EWS* gene in normal mouse tissues. Lanes 1 to 12 represent (1) bladder, (2) tongue, (3) pancreas, (4) ovary, (5) stomach, (6) thymus, (7) spleen, (8) small intestine, (9) heart, (10) kidney, (11) brain, and (12) lung respectively.

was smaller than the original *EWS* gene with various premature stop codons observed (Fig.6.4A and Fig.6.4B). Moreover, both pseudogenes have different mutations and deletions but with high nucleotide sequence similarity. This indicates that there is more than one *EWS* pseudogene in the mouse genome. Specific primers were designed and used for RT-PCR on ES cell cDNA library revealed that there were no pseudogene transcripts observed (data not shown), indicating that these pseudogenes are not transcribed.

6.2-3 Isolation of Murine *EWS* Genomic Fragments

Since the presence of murine *EWS* pseudogenes hindered the isolation of appropriate genomic clones from genomic library screening, an alternative approach to obtain murine *EWS* genomic fragments is using *Elongase* PCR on 129S6/SvErTac ES cell DNA to amplify all murine *EWS* genomic fragments in this study. In order to avoid the non-specific amplification, specific primers used in *Elongase* PCR were designed according to the non-overlapping regions between murine *EWS* cDNA and its pseudogenes and intronic regions (primers used see Appendix 4). Firstly, a PCR fragment with 1.8 kb from exons 2 to 4 and another fragment with 4.0 kb from exons 6 to 8 were successfully amplified and analyzed on agarose gel using MEWSN-AS/MEWSN-DA and MEWS-6S/MEWS-8A primers respectively (Fig.6.5B). The *Elongase* PCR fragments were added dATP by a 72^0C for 10 minutes extension reaction using *Taq* polymerase. After purification, these PCR fragments were subcloned into pGEMT vector and inserts were sequenced. Specific primers were designed according to the intronic sequences of introns 3 and 7 (MEWSINT-3S and MEWSINT-7S) (see Appendix 4). The second genomic fragments of a 4.2 kb fragment from intron 3 to exon 5 (MEWSINT-3S/MEWS-5A)) and another 3.8kb fragment from intron 7 to exon 10 (MEWSINT-7S/MEWS-10A) which were also subcloned into the pGEMT vector (Fig.6.5B). These fragments were verified by sequencing using T7 and SP6 primers (data not shown). These four genomic fragments were used for the generation of both *EWS* gene knockout and *EWS/Fli-1* fusion gene knock-in constructs, which are discussed in the following chapters.

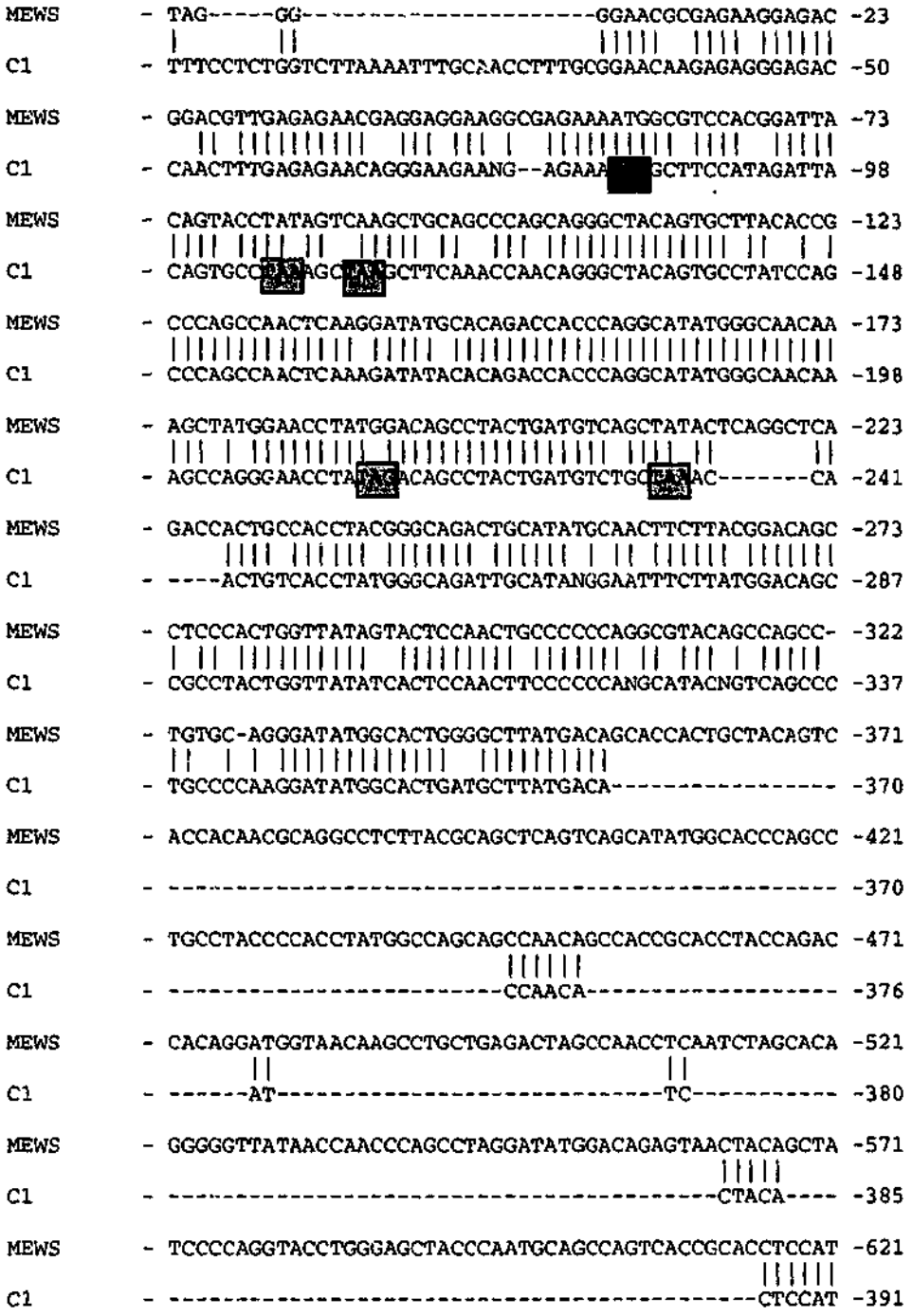


Fig.6.4A Nucleotide sequence alignment between murine *EWS* cDNA and the murine EWS pseudogene, C1, which was isolated from a 129SvJ gFIXII genomic library (Stratagene). The green box represents the initiation codon while the red boxes are stop codons.

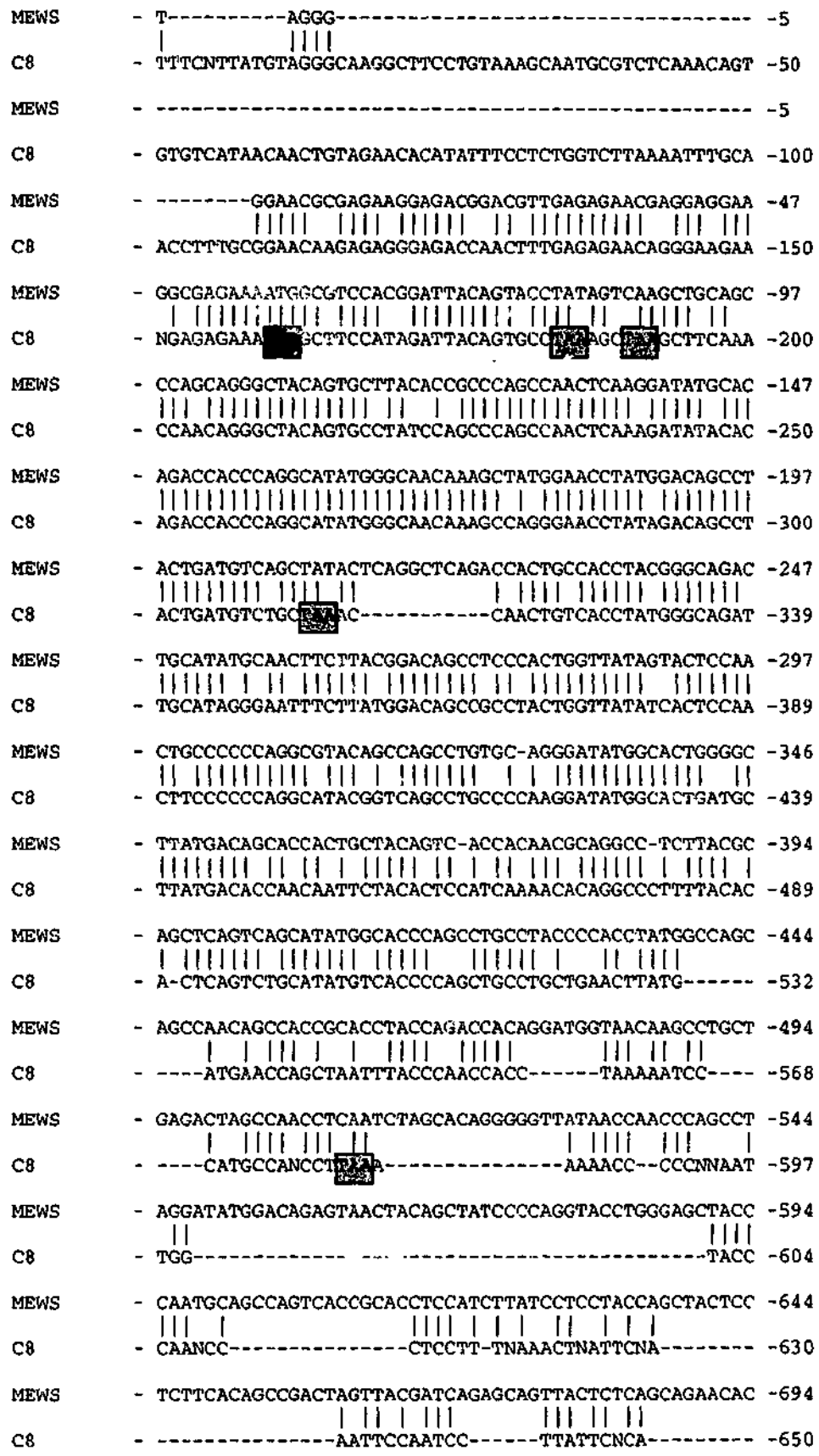


Fig.6.4B Nucleotide sequence alignment between murine *EWS* cDNA and the murine EWS pseudogene, C8, which was isolated from a 129SvJ gFIXII genomic library (Stratagene). The green box represents the initiation codon while the red boxes are stop codons.

Other genomic fragments of murine *EWS* were amplified such as a 5.3kb fragment was amplified from exons 1 to 2 by MEWS 1SB/MEWSINT-2A and a 3.5kb fragment was amplified from exons 5 to 6 by MEWSI-4S/MEWS-6A. All these genomic fragments were subcloned and sequenced and their exon/intron boundaries are defined and fitted the Breatnach (GT/AG) rule (Breatnach *et al*, 1978) (data not shown).

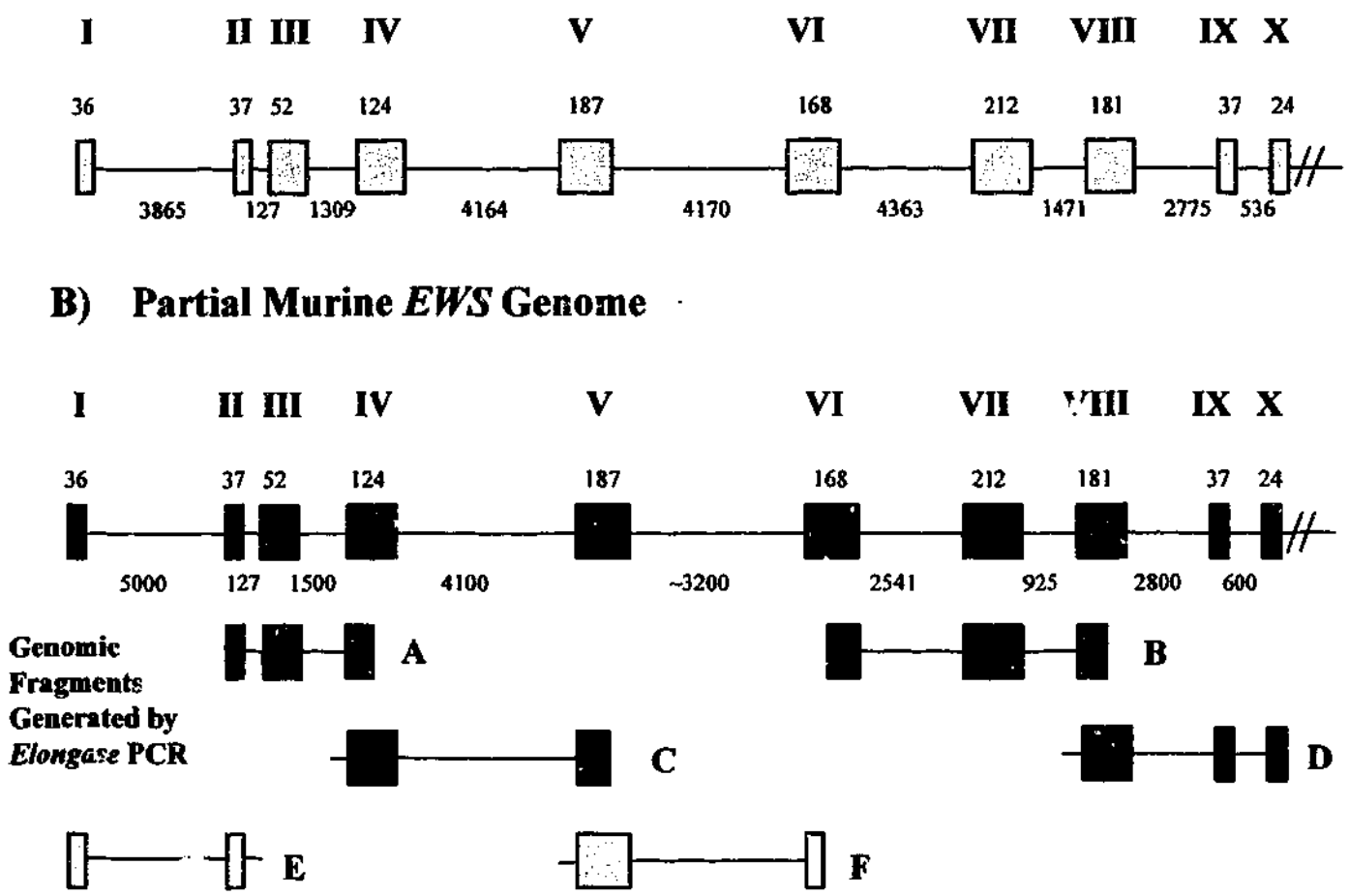
The open reading frame of *EWS* gene between mouse and human is identical and the genomic structure of murine *EWS* is similar to human *EWS* except different intronic sizes between murine and human *EWS* gene (Fig.6.5A). The murine *EWS* intron 1 is ~1200bp longer than human (mouse is 5000bp and human is 3865bp) (Fig.6.5A). However, introns 5, 6 and 7 of human *EWS* are longer than murine *EWS* (Fig.6.5A).

6.2-4 Comparison of Human and Murine *EWS* Introns 6 and 7

The introns 6 and 7 of human *EWS* are the commonest regions for translocation in Ewing's sarcoma (Zucman *et al*, 1993). Sequence analysis on murine *EWS* introns 6 and 7 found that there were regions with significant homology between these regions.

Sequence of murine intron 7 revealed that there are neither similar consensus sequence such as 5'-AGAAAARDRR-3' nor eukaryotic topoisomerase II cleavage sites reported in previous report for human *EWS* (Obata *et al*, 1999). However, some high identical repeats in intron 7 of murine *EWS* such as 5'-AGAAAACTAA-3', 5'-AGAAAAATCC-3' OR 5'-AGAAGGG-3' were found and their patterns were similar to the reported repeat sequences in human *EWS* (Obata *et al*, 1999). Additionally, there are many *Alu* repeats found in murine *EWS* intron 7 especially at the middle position (data not shown). According to the FASTA results, the most dominant *Alu-like* repeat is *Alu-J* and other *Alu-like* repeats also consists of *Alu-Sx* and *Alu-Sp* (data not shown). There were two sites in murine *EWS* intron 7 showed highly identity to intron 7 of human *EWS* (Fig.6.6). These two sequences located at 233 bp and adjacent to exon 8 of both murine and human *EWS* respectively (Fig.6.6).

In mouse and human intron 6, there were many *Alu-like* repeats distribute at both ends of intron 6 of murine *EWS* while the *Alu* repeats distribute evenly in human *EWS* intron



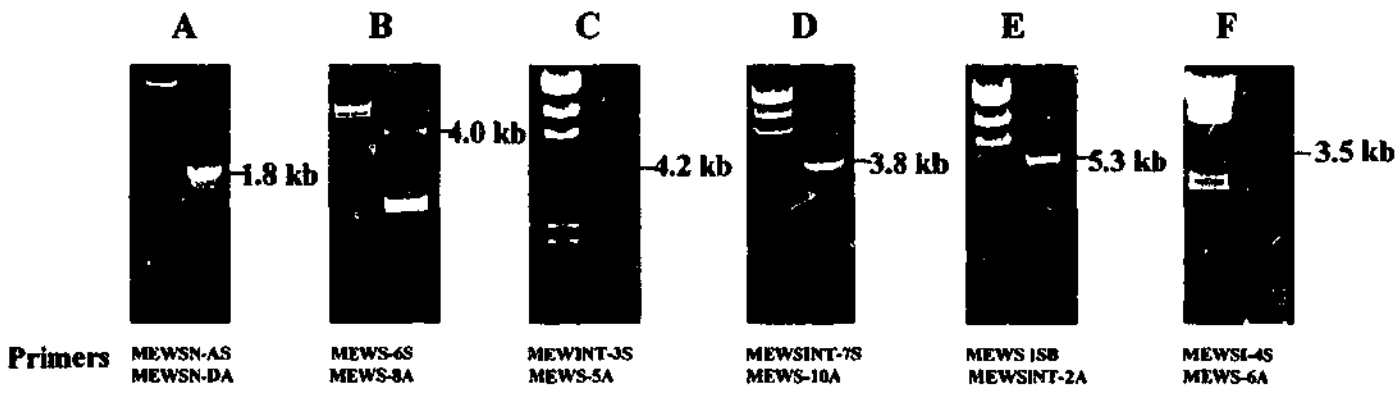


Fig.6.5 Schematic diagrams show the partial genomic structure (exons 1-10) of the human and murine *EWS* genes. (A) Partial human *EWS* genome. (B) Partial murine *EWS* genome and the Elongase PCR amplified fragments used for knockout and knock-in constructs. The exons are shown to scale as solid boxes. The lines represent introns and the numbers represent base pair (bp). The photos show the amplified genomic fragments on agarose gel.

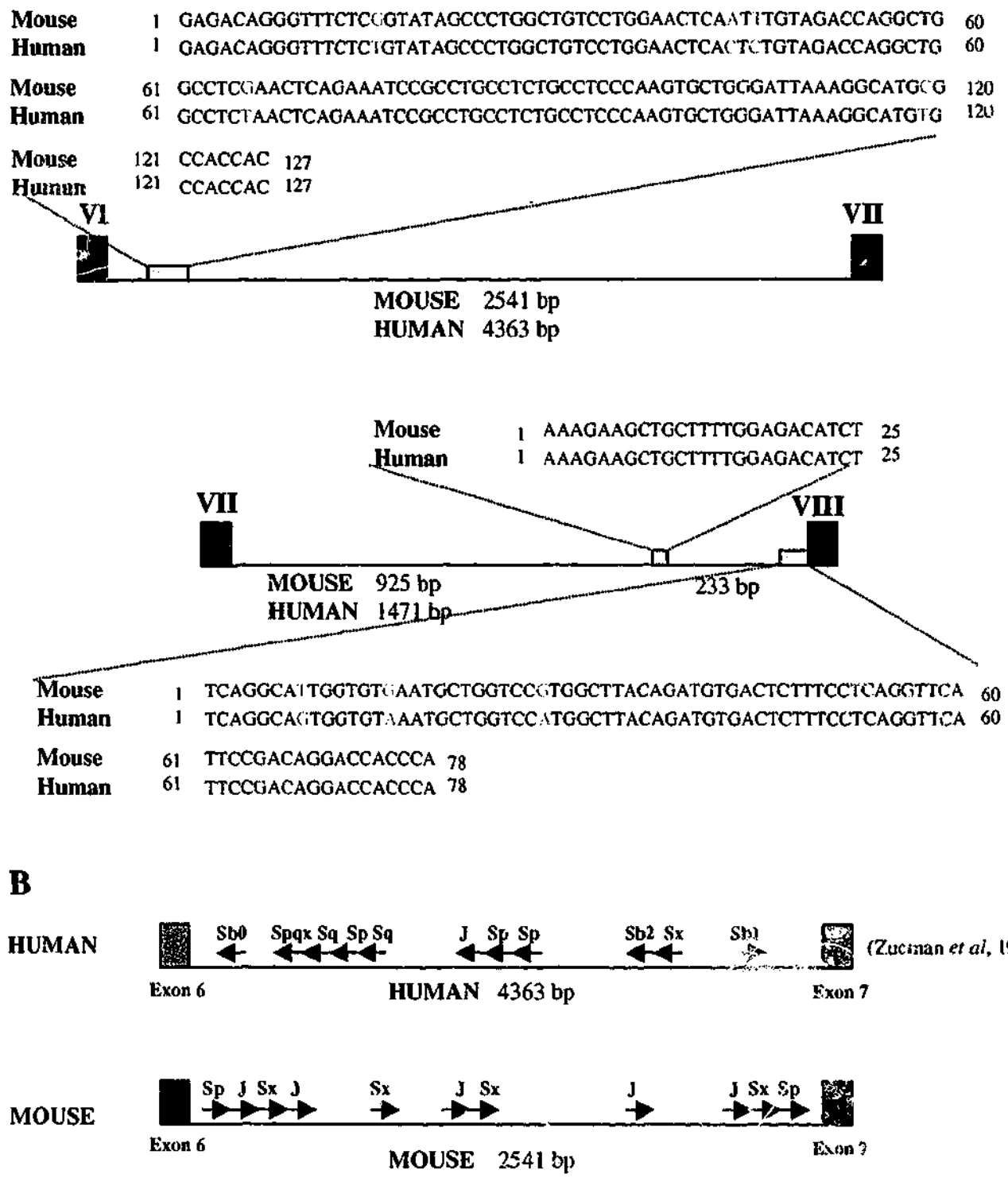
6. Similar to intron 7, there was one region (127 bp) located at 99 bp from exon 6 and showed highly identity between human and mouse (Fig.6.6). The function of these high identity regions is unclear, but could be translocation or exon splicing signals. However, the similarity of this intron between mouse and human may provide a basis for investigation of the fragility in this intron for translocations in the human.

6.2-5 Generation of *EWS* Gene Knockout and *EWS/Fli-1* Fusion Gene Knock-in Constructs

Several *EWS* gene knockout and *EWS/Fli-1* knock-in constructs were initially generated using conventional targeting strategy using either *pMC1-Neo* (1.1kb) or *pGK-Neo* (1.8kb) as a positive selection marker (data not shown). The *EWS* gene knockout was designed to target at exons 4 and 5 of *EWS* which will delete the normal biological functions of *EWS*. For the *EWS* knockout constructs, exons 2 to 4 (1.8 kb) or exons 4 to 5 (4.0 kb) as the 5' homologous arm and intron 4 (2.0 kb) and intron 7 to exon 8 (2.0 kb) as the 3' homologous arm (data not shown). For the *EWS/Fli-1* knock-in constructs, exons 6 and 7 (2.8 kb) as the 5' homologous arm and intron 7 to exon 8 (2.0 kb) as the 3' homologous arm with *Fli-1* EBD cDNA in-framed to exon 7 of *EWS* (data not shown). Electroporation of these constructs generated large numbers of surviving clones. Thirty μg of construct DNA electroporated into 2 x 10^7 ES cells resulted in 900 and more than 1000 surviving clones for knockout and knock-in constructs respectively after 1 week G418 selection. However, no targeted clones were obtained. This may be due to random insertion of knockout constructs into the mouse genome.

Since the *EWS* gene is ubiquitously expressed with a 3.0 kb transcript in ES cells, a promoterless *Neo* from *IRES/Neo* gene cassette (a gift from Dr. P Mountford, MIRD, Monash University, Australia) was used as a positive selection marker. The 1.8 kb *IRES/Neo* with poly (A) tail was replaced the pGK-Neo in both *EWS* knockout and *EWS/Fli-1* knock-in constructs (Fig.6.7 and Fig.6.8). The 5' homologous arms consist of exons 4 to 5 (4.0 kb) for *EWS* knockout construct and exons 6 and 7 (2.8 kb) for *EWS/Fli-1* knock-in construct (Fig.6.7 and Fig.6.8). The 3' homologous arm for both constructs includes intron 7 to exon 8 (2.0 kb). Maps and verification of constructs by restriction enzyme digests and PCR are shown in Fig.6.7 and Fig.6.8.

A

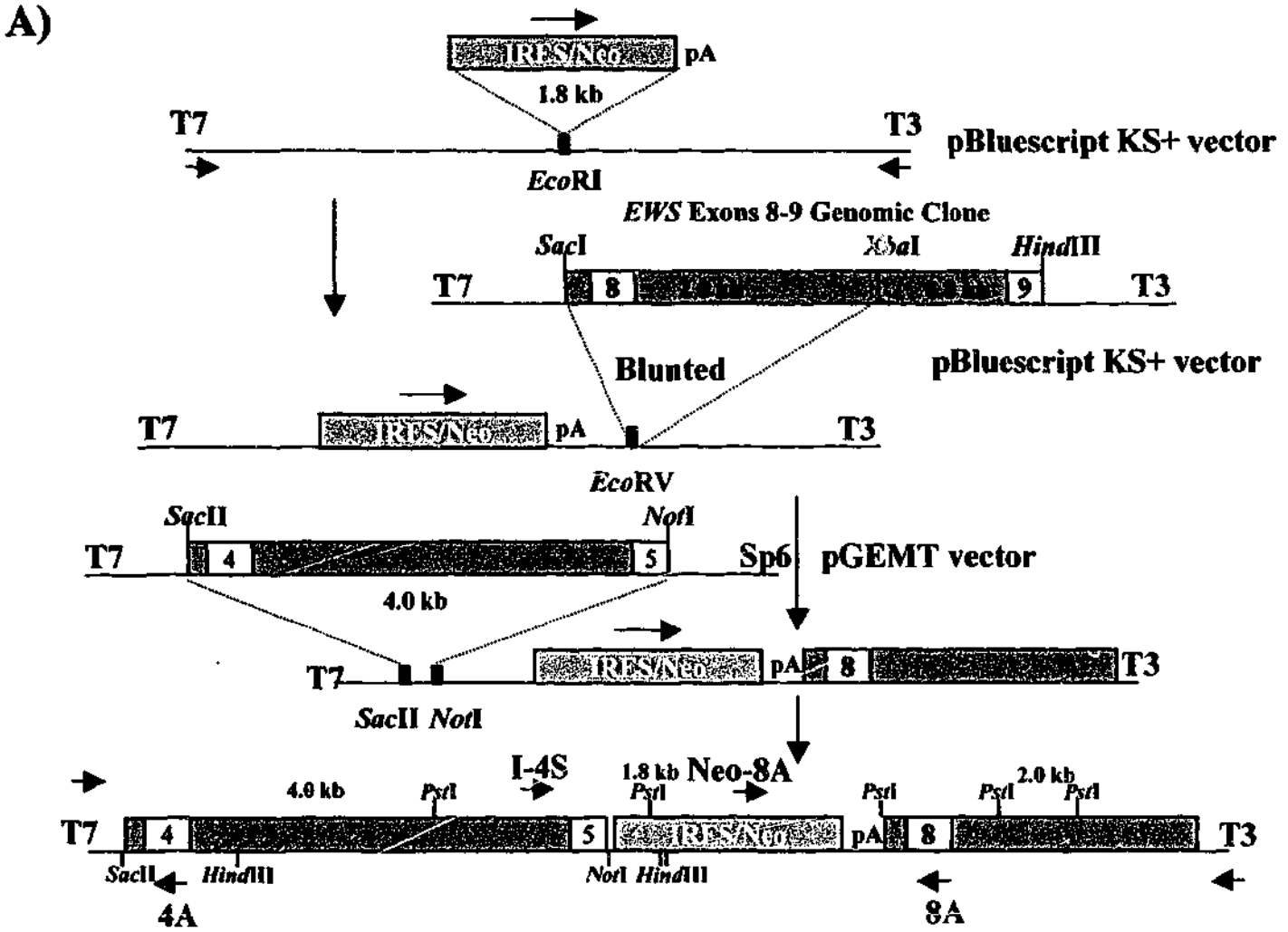


B

Fig.6.6 Comparison of intronic similarities between human and mouse *EWS* introns 6 and 7. (A) The comparison of sequence of some high identity regions in mouse and human *EWS* introns 6 and 7. (B) Distribution of the *Alu-like* B1 repeats and *Alu* repeats in mouse and human *EWS* intron 6 respectively (human data was extracted from Zucman *et al*, 1997).

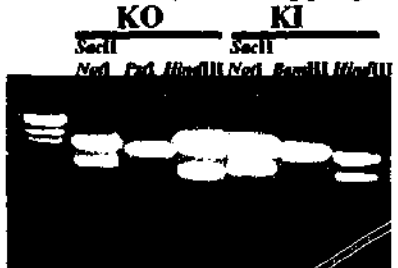
Fig.6.7 Generation of *EWS* gene knockout construct. (A) Steps of constructing *EWS* gene knockout targeting vector. A 1.8kb promoterless Neomycin selection marker, TRES/Neo, was cloned into *Eco*RI site of pBluescript KS+ vector. A 2.0 kb 3' homologous arm including exon 8 was cloned into *Eco*RV site with blunt-end ligation. The 5' homologous arm (4.0 kb) including exons 4 and 5 was cloned into *Sac*II and *Not*I site. (B) Verification of *EWS* knockout construct by restriction enzyme mapping and *Taq* PCR using different combinations of primers. The KO represents *EWS* knockout construct and KI represents *EWS/Fli-1* knock-in construct showed at Fig.6.8. (C) Restriction map of the wild-type *EWS* locus (partial) and targeting vector showing the targeting and screening strategy for *EWS* knockout ES clones. 3' external probe (0.8 kb) and 5' internal probe (0.6 kb) were used after digesting with *Hind*III and *Bam*HI respectively.

A)

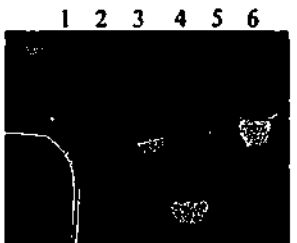


B) **Verification of *EWS* Knockout Construct**

Restriction Enzymes Mapping

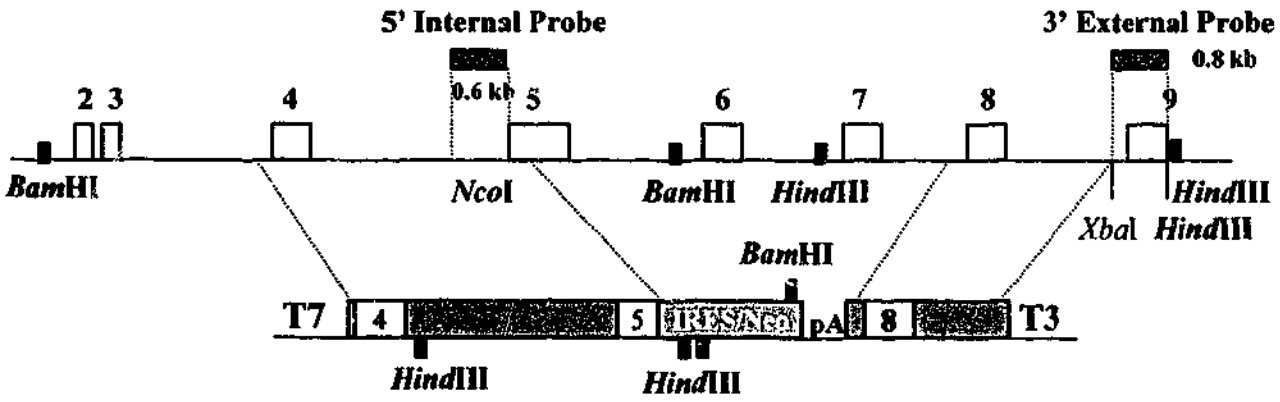


***Taq* PCR**



Primers	Size
1. T7/4A	230 bp
2. T3/4A	none
3. I-4S/8A	2.25 kb
4. Neo-2/8A	1.0kb
5. Neo-2/T7	none
6. Neo-2/T3	2.7 kb

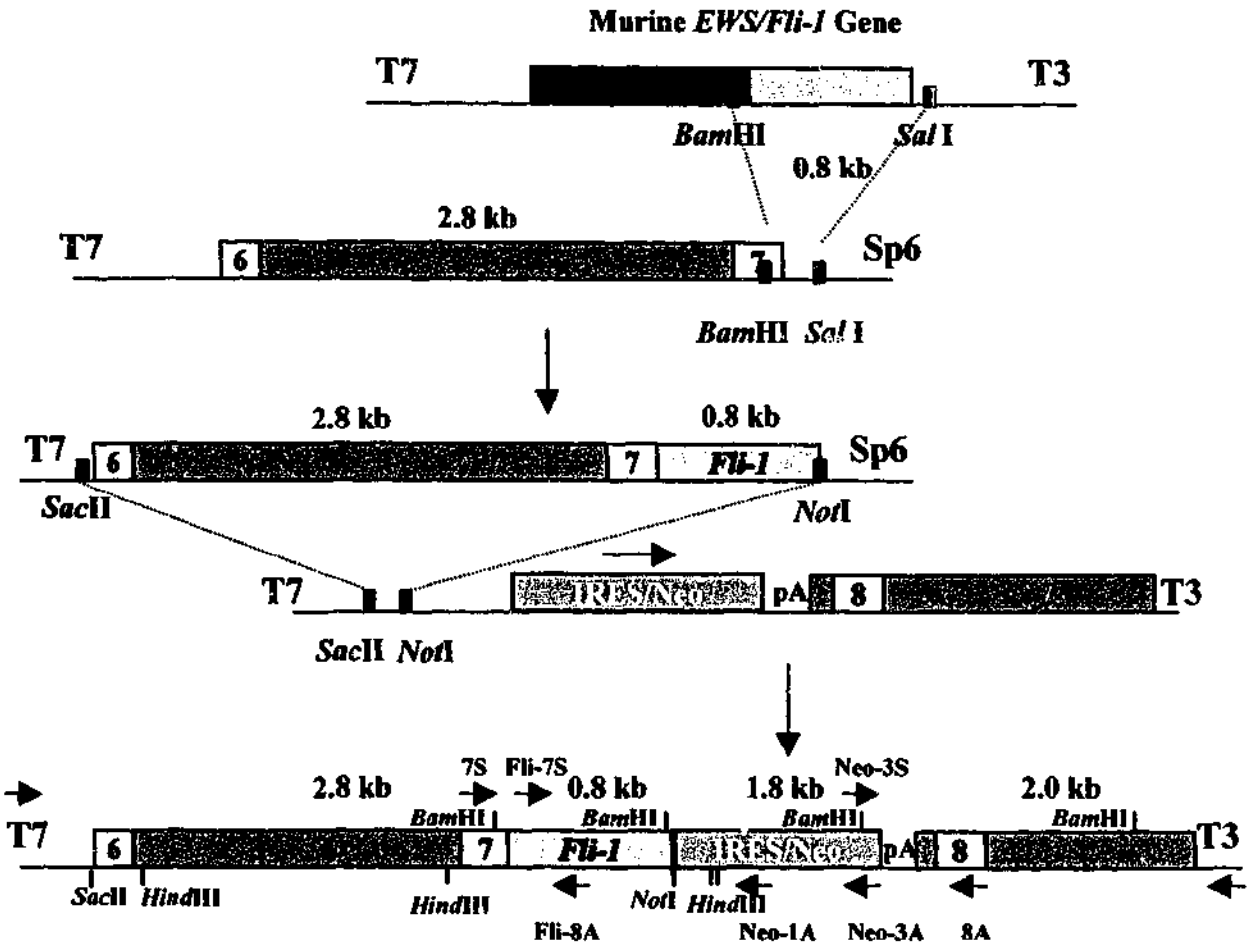
C)



***EWS* Knockout Targeting Vector**

Fig.6.8 Generation of *EWS/Fli-1* knock-in construct. (A) Steps of constructing EWS/Fli-1 knock-in targeting vector. An in-frame fuse murine *EWS/Fli-1* used in Chapter 3 was digested by *Bam*HI (inside exon 7) and *Sal*I to release a 0.8 kb fragment which was fused to the *Bam*HI and *Sal* I sites of a genomic clone (*EWS* exon 6 to Exon 7) in pGEMT vector. The fusion point was sequenced for confirmation. This fusion fragment (3.6 kb) was cloned into *Sac*II and *Not*I sites of a pBluescript vector which has already had IRES/Neo and 3' homologous arm. (B) Verification of *EWS/Fli-1* knock-in construct using different combination of Primers in *Taq* PCR. (C) Restriction map of the the wild-type *EWS* locus (partial) and targeting vector showing the targeting and screening strategy for *EWS/Fli-1* knock-in ES clones. 3' external probe (0.8 kb) and the *Fli-1* partial cDNA probe ((0.8 kb internal probe) were used after digesting with *Hind*III and *Eco*RI respectively.

A)

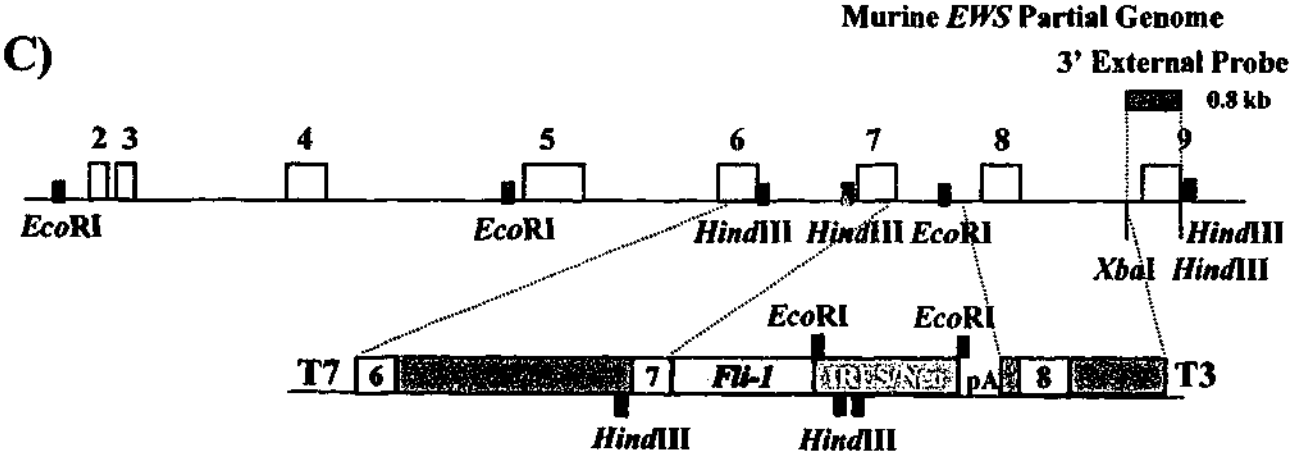


B) **Verification of *EWS/Fli-1* Knock-in Construct**

***Taq* PCR**

Primers	Size
1. T7/Fli-8A	2.8 kb
2. 7S/Fli-8A	0.2 kb
3. 7S/Neo-1A	2.1 kb
4. Fli-7S/Neo-3A	2.4 kb
5. Neo-3S/8A	0.3 kb
6. Neo-3S/T3	2.0 kb

C)



***EWS/Fli-1* Knock-in Targeting Vector**

6.2-6 The Specificity of 3' External and 5' Internal Probes

To screen for correctly positive targeted ES clones, Southern blot analysis on ES cell genomic DNA was used. Specific external or internal probes were necessary for Southern blot analysis.

BLAST analysis of genomic fragments outside the 5' or 3' homologous regions used for targeting indicated the presence of repetitive sequences unsuitable for specific binding on Southern blots (data not shown). Thus, a 0.8 kb region avoiding these elements was excised (*Xba*I and *Hind*III) from the *EWS* genomic fragment (Fig.6.7C, Fig.6.8C). This was used as an external probe and showed high specificity in Southern blot analysis of knockout and knock-in clones (Fig.6.10). Another internal probe which was prepared by *Nco*I digestion also showed high specificity and was used for knockout clones screening (Fig.6.7C, Fig.6.10).

6.2-7 Screening Strategies for Knockout and Knock-in Clones

When using the 3' external probe for screening knockout and knock-in clones, *Hind*III restriction enzyme was used to digest 10 μg genomic DNA (~20 μl) for 4 hours or overnight at 37^0C. A 7.0 kb band was detected in wild-type ES cell genomic DNA (Fig.6.8C and Fig.6.9) while a 4.5 kb band should be observed in the correctly targeted knockout or knock-in clones.

The 5' internal probe was suitable for confirming knockout clones, since after *Bam*HI digestion, this probe detects a 9.8 kb wild-type band, however a 7.8 kb band should also be detected in targeted clones (Fig.6.8C).

To confirm knock-in clones, a *Fli-1* EDB domain cDNA (0.75 kb) would be suitable, since after *Eco*RI digestion, the knock-in clones should display a 7.2 kb targeted band (Fig.6.8C).

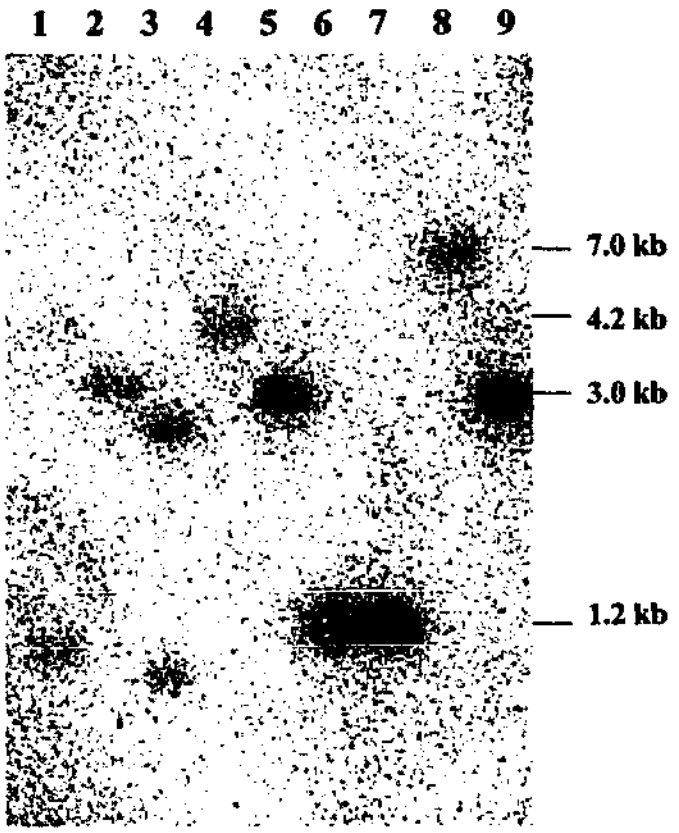


Fig.6.9 Test of 3' external probe on wild type ES cell genomic DNA on Southern analysis. Lanes 1-9 represent *Apa*I, *Bam*HI, *Apa*I/*Bam*HI, *Sac*I, *Sac*I/*Bam*HI, *Xba*I, *Xba*I/*Sac*I, *Hind*III and *Hind*III/*Sac*I restriction digestions.

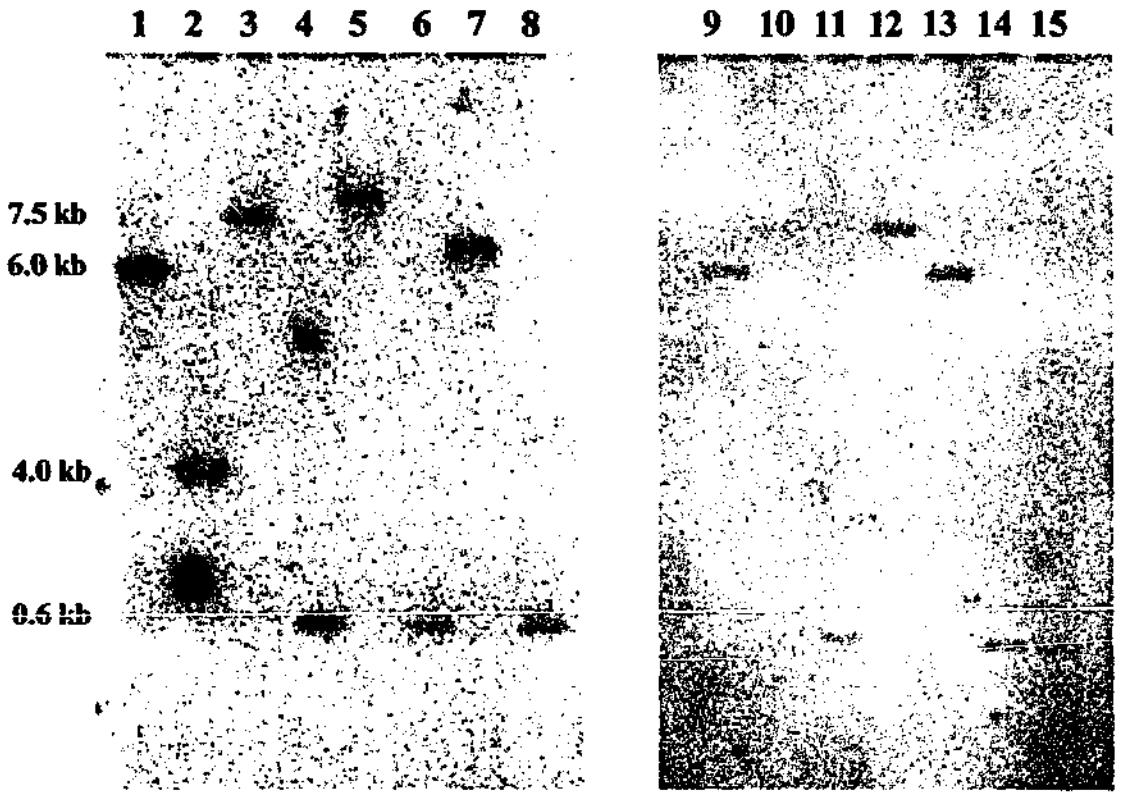


Fig.6.10 Test of 5' internal probe on wild type ES cell genomic DNA on Southern analysis. Lanes 1-15 represent *Eco*RI, *Eco*RI/*Hind*III, *Eco*RV, *Eco*RV/*Nco*I, *Apa*I, *Apa*I/*Nco*I, *Hind*III, *Hind*III/*Nco*I, *Sac*I, *Sac*I/*Nco*I, *Bgl*I, *Bgl*I/*Nco*I, *Bgl*II, *Bgl*II/*Nco*I and *Nco*I restriction digestions.

Discussion:

In this study, the expression patterns and genomic structure of murine *EWS* were investigated. The expression patterns of both murine and human *EWS* were found to be similar. Furthermore, pseudogenes for *EWS* have been identified in the mouse genome similar to that shown in the human genome and both species have repetitive elements located in introns 6 and 7. Thus *EWS* is highly conserved and murine *EWS* may possess similar functions to human *EWS*.

The expression pattern of murine *EWS* was shown to be similar to human *EWS*, ubiquitously expressed in both human or mouse normal tissues. However, this study did not identify the alternative spliced transcripts of murine *EWS* as described for human *EWS* (Ohno *et al*, 1994; Aman *et al*, 1996). The murine *EWS* transcript was shown to be longer than human *EWS* transcript, suggesting that the murine *EWS* has longer 3'-UTR or poly A tail. The widespread expression of both murine and human *EWS* may due to the high content of unmethylated CpG dinucleotides at the 5' region of the *EWS* gene, a common feature of the promoter region for many "housekeeping genes" (Bird, 1986; Zucman *et al*, 1993). "Housekeeping genes" are expressed in every cells and play very important roles in normal cellular functions and development of organism (Jolly, 1986; Nothias *et al*, 1995; Luft *et al*, 1999). Thus, EWS may also play important role in normal cellular functions.

During the identification of *EWS* genomic phage clones, a number of clones containing *EWS* reactive genomic inserts were isolated and sub-cloned. However, sequence analysis revealed that these clones had high identity to murine *EWS* exons 1 to 7 without introns or poly A tail at the 3'-end and included many mutations and stop codons. All these features are common to traditional pseudogenes (Nouvel, 1994) and were consistent with the description of human *EWS* pseudogenes (Bovee *et al*, 1994). Pseudogenes originate from retrotransposition (Nouvel, 1994) and therefore, the human and murine *EWS* pseudogenes are believed to be derived from *EWS* mRNA that has been converted into DNA and inserted into the genome. During evolution, the percentage of homology to the cDNA of the original gene decreases and thus they do not encode functional proteins. A wide range of genes, especially "housekeeping

genes", have been shown to have one or several retrotransposed homologs in the mammalian genome such as human ferrochelatase (Whitcombe *et al*, 1994), dihydrolipoyl succinyltransferase (E2K) (Cai *et al*, 1994) and glyceradehyde-3-phosphate dehydrogenase (Foss *et al*, 1994). Hence, the *EWS* gene may be an evolutionary conserved, fundamentally important gene in the mammalian genome.

The presence of *EWS* pseudogenes in mouse genome hindered the isolation of true *EWS* genomic fragments by genomic library screening. Thus, in order to obtain genomic *EWS* fragments, a PCR approach using *Elongase* to amplify these fragments from mouse ES genomic DNA was applied. *Elongase* contains a mixture of *Taq* and *Pyrococcus* species GB-D thermostable DNA polymerase, an enzyme with 3'→5' exonuclease activity, which has proofreading function for PCR fragments. To avoid from amplifying the pseudogenes in mouse genome, specific primers were designed and used to amplify the genomic fragments of exons 2-4 and exons 6-8. Other genomic fragments were amplified by some specific primers designed according to the intronic sequences of sequences of exons 2-4 and exons 6-8. The sizes of all the exons were identical between murine and human *EWS* as they have similar open reading frame. The size of each introns was slightly different except the intron 6 where the size of murine has ~2-fold less than human.

Introns 6 and 7 of human *EWS* are the most common site for translocation with the *FLI-1* gene creating the types I and II *EWS/FLI-1,* which are the dominant pattern in Ewing's sarcoma (May *et al*, 1993; Zucman *et al*, 1993; Delattre *et al*, 1994; Bhagirath *et al*, 1995). Due to the highly conserved nature of this gene, it is interesting to compare these introns of mouse with human to examine any structural regions in favor for translocations. Murine introns 6 and 7 showed a very high density of *Alu-like* repeat sequences similar to human *EWS* intron 6. *Alu* retroposition and recombination is believed to enhance the genome plasticity for chromosomal breakage (Zucman *et al*, 1997). The finding of a smaller 1.8 kb intron 6 in one allele of human *EWS* in individuals of African origin explains the lowest incidence of Ewing's sarcoma and indicates that the different rate of racial incidence of Ewing's sarcoma depends on the size of introns. (Zucman *et al*, 1997). The size of murine *EWS* intron 6 is almost 2-fold less than human intron 6. Thus, this may explain why chromosomal translocation in these introns of *EWS* in mouse has not been observed. In addition, this study also found

some intriguing patterns in introns 6 and 7 of murine *EWS* where 3 high identity regions were found near the murine and human *EWS* exon 8. These regions were found in murine and human *EWS* introns 6 and 7 and not other introns or other genes. However, the function or significance of these regions is unknown.

After characterization of the murine *EWS* genomic structure, these genomic fragments were used to generate *EWS* knockout and knock-in constructs. These will enable the generation of mice to analyse normal EWS function *in vivo* and mimic Ewing's sarcoma respectively. The gene targeting technology in either knockout or knock-in strategies involves the recombination between DNA sequences at a specific genomic locus and newly introduced DNA sequences that are highly similar (Thomas and Capecchi, 1987). The low targeting efficiency observed in the knockout and knock-in strategies may be due to the following reasons. Firstly, the specificity of homologous arms may affect the efficiency of homologous recombination and correct targeting locus. In this study, the number of surviving clones in knock-in targeting was much higher than the knockout. This may be due to the less specific 5' homologous arm of *EWS/Fli-1* knock-in vector which contains many *Alu-like* repeats in *EWS* intron 6. However, this region is necessary to fuse *EWS* exon 7 to *Fli-1* EDB domain to generate the *EWS/Fli-1* fusion gene and cannot be changed. In addition, the low targeting efficiency of *EWS* might also be due to the location of *EWS* in the genome. Some regions of the genome may have extensive tertiary structures and thus not as accessible to the targeting vectors. Moreover, the targeting vectors may insert randomly into the genome. The uses of conventional positive selection markers such as *pMC1-Neo* and *pGK-Neo* will result in these cells with non-specific insertions to be resistant to G418 selection.

Thus, to improve the targeting efficiency and reduce the number of background survival clones, a promoterless *IRES/Neo* cassette was used. The advantage of this promoterless *IRES/Neo* cassette is that the expression of *Neomycin* gene depends on the targeted gene promoter. This might reduce the number of non-specific clones raised from non-specific recombination and randomly integration (Doetschman *et al*, 1988; Schwartzberg *et al*, 1990; Macleod *et al*, 1991; Salminen *et al*, 1998). The limitation of using this cassette in the targeting vector is that the targeted gene needs to be expressed in ES cells (Macleod *et al*, 1991; Salminen *et al*, 1998), however of murine *EWS* gene is expressed in mouse 129S6/SvErTac and C57B1/6J embryonic stem (ES) cell lines.

Although the targeted ES clones and mice haven't yet been obtained, the phenotypes of the *EWS* knockout and knock-in mice may be predicted by recent reports in studying of *TLS/FUS* knockout and *TLS/CHOP* knock-in mice (Kuroda *et al*, 1999, 2000; Hicks *et al*, 2000). Similar to EWS, TLS/FUS belongs to a novel sub-family of the TET family of RNA-binding proteins, expressed ubiquitously and involved in translocations of many human cancers (Crozat *et al*, 1993; Prasad *et al*, 1994; Zinszner *et al*, 1994; Aman *et al*, 1996; Panagopoulos *et al*, 1997). TLS/FUS deficient mice demonstrated abnormalities in B-lymphocyte development, chromosomal stability and DNA pairing (Kuroda *et al*, 2000; Hicks *et al*, 2000). In addition, mice with TLS/CHOP fusion expression developed a liposarcoma-like phenotype (Kuroda *et al*, 1999; Perez-Losada *et al*, 2000). Thus, the generation of mouse model with Ewing's sarcoma using the knock-in approach is feasible.

Chapter 7

Summary and Conclusions

This thesis describes the ability of murine and human EWS/FLI-1 to transform and the effect of an engineered specific transcriptional repressor, KRAB/FLI-1, on these transformed cells. Importantly, this repressor specifically suppressed EWS/FLI-1 activated genes and reversed the transformed phenotypes of these cells and human ES/PNET cells. These data indicate that this transcriptional repressor may provide a potential approach for gene therapy of ES/PNET and that these cell lines provide excellent reagents to further elucidate the biological mechanisms of EWS/FLI-1 oncogenes.

This thesis also describes the isolation of a novel ETS transcription factor which is turned on in expression in both ES/PNET and a wide range of human cancers. Continuing work will further characterise this gene and also complete the development of two mouse models initiated during this study. These models will be an *EWS* gene knockout and *EWS/Fli-1* knock-in mice which will provide *in vivo* knowledge of the normal function of *EWS* gene and the aetiology of Ewing's sarcoma respectively.

7.1 Transformation on NIH3T3 by Murine *EWS/Fli-1* Fusion Gene

The majority of Ewing's sarcoma (ES) and primitive neuroectodermal tumours (PNET) have a t(11;22)(q24;q12) chromosomal translocation which fuses the amino terminus of *EWS* gene to the carboxyl terminus of *FLI-1* gene (Zucman *et al*, 1993; Sorensen *et al*, 1994), suggesting that this fusion protein plays a key role in the pathogenesis of these tumours. To further understanding the biological mechanisms of this fusion gene, a human and a murine *EWS/Fli-1* fusion gene which mimic the human fusion gene generated by the ES/PNET type I translocation. These genes showed 90% nucleotide and >95% amino acid identities. Stable expression of these genes in NIH3T3 cells demonstrated similar tumourigenicity by anchorage-independent growth in soft agar,

high proliferation rate in low serum medium and tumour development in *BALB/c nu/nu* mice. These data demonstrated that the potential for development of a murine model for ES/PNET. Interestingly, the levels of the fusion protein expression correlated with the colony formation efficiency of EWS/FLI-1 in the soft agar assay. This finding was consistent with previous data which showed that the proliferation rate of SK-N-MC is much faster than SK-ES-1 and the EWS/FLI-1 expression level in SK-N-MC has been showed to be 23 -folds more than SK-ES-1 (Tanaka *et al*, 1997). This may indicate that the expression level of EWS/FLI-1 onco-protein can affect tumourigenic potential.

Furthermore, to determine whether some of the putative downstream target genes activated in ES/PNET tumour cells were also altered in human and murine EWS/FLI-1 transformed NIH3T3 cells, the expression levels of *c-myc*, *Stromelysin-1*, *EAT2* and *Manic Fringe* genes were studied by Western blot, semi-quantitative RT-PCR or real-time quantitative RT-PCR analysis. Levels of these genes were elevated in *EWS/FLI-1* transfected cells compared with mock-transfected controls. These data were similar to other reports which showed that EWS/FLI-1 transformed cells have dysregulation of a number of genes which as potentially FLI-1 targets (or downstream thereof) (see review in Section 1.5-3).

7.2 EWS/FLI-1 Induced Transformation is Reversed by a KRAB/FLI-1 Suppressor Protein

Although the precise mechanism by which EWS/FLI-1 causes tumourigenesis in ES/PNET is still unclear, many reports have demonstrated that this chimaeric fusion protein can modulate transcription of specific genes associated with oncogenesis. These putative oncogenes, in turn, can activate numerous signalling pathways resulting in ES/PNET. Several known pathways are altered in ES/PNET such as the *IGF-IR* signalling pathway (Toretsky *et al*, 1999), phospholipase C signaling pathway (Dohjima *et al*, 2000), *bFGF* pathway (Girnita *et al*, 2000) and the mitogen-activated protein kinase (*MAPK*) signalling pathway (Silvany *et al*, 2000). All these pathways have been shown to be important in ES/PNET and are closely related to the presence of EWS/FLI-1 fusion protein. The DNA binding domain of EWS/FLI-1 appears to be an important

determinant in the generation of ES/PNET as the fusion protein acts as a transcription factor which specifically recognises the ETS sites within gene promoters. There is evidence that the transcriptional repressors, KRAB/DNA-binding-domain fusion proteins, can efficiently suppress promoter-reporter constructs containing specific DNA recognition sequences (Beerli *et al*, 1998; Herchenroder *et al*, 1999; Ma *et al*, 1999; de Haan *et al*, 2000), and similar fusions inhibit tumour formation or transformation phenotypes *in vitro* and *in vivo* (Rossi *et al*, 1999; Fredericks *et al*, 2000; Nawrath *et al*, 2000; Ayyanathank *et al*, 2000). Thus, the FLI-1 DNA binding domain was fused to this transcriptional repressor, KRAB, to the promoters of genes potentially bound and upregulated by EWS/FLI-1.

Stable expression of *KF* fusion gene in EWS/FLI-1 transformed NIH3T3 cells resulted in loss of the ability of EWS/FLI-1 transformed cell lines to grow in low serum medium and soft agar, and impairment of tumour formation in *BALB/c nu/nu* mice. In addition, introduction of a *KF* fusion gene to a human PNET cell line, SK-N-MC, resulted in similar growth retardation *in vitro* and *in vivo*. To assess whether genes shown to be upregulated in ES/PNET (and transformed NIH3T3 cells) were suppressed by KF, genes such as *Stromelysin-1*, *Manic Fringe*, *EAT-2* and *c-myc* were examined by either Western, semi-quantitative RT-PCR or real time quantitative RT-PCR analysis. These data revealed that the expression of these genes was repressed. The *c-myc* gene has a critical role in the control of cellular proliferation (Henriksson *et al*, 1996) and dysregulated c-myc has been associated with a variety of tumours including ES/PNET (Bailly *et al*, 1994). The results in this thesis demonstrated that the c-myc expression level was higher in EWS/FLI-1 transformed NIH3T3 and SK-N-MC cell lines but reduced in *KF* co-transfectants. These correlated the alteration of the malignant growth properties of EWS/FLI-1 transformed NIH3T3 and SK-N-MC cells after expression of the KF repressor. Further, the relative levels of EWS/FLI-1 to KF appeared to be important for both alterations in gene transcription and the transformation phenotype. In ES/PNET cells, SK-N-MC, *c-myc* expression was only slightly repressed, however the suppression effect on the malignant phenotypes was significant. These data indicate that many factors which may contribute to the malignant phenotypes are regulated by EWS/FLI-1 and KF and suggest that further examinations of these cell lines will be valuable in the elucidation of the aetiology of ES/PNET.

7.3 Isolation of A Novel ETS Gene, *ER99*, Expressed in ES/PNET

This thesis also describes the isolation of a novel ETS gene termed ER99. Although this factor was originally isolated from a human breast cancer cell line by degenerative PCR, this was demonstrated to be overexpressed in ES/PNET cell lines and in EWS/FLI-1 transformed fibroblasts. Screening of human testis cDNA library and 5' RACE PCR failed to generate a full-length cDNA, however a 400 bp partial cDNA was amplified by 3' RACE PCR. By bioinformative analysis, this fragment was shown to be transcripted from human chromosome 19q13.1 and similar to murine ER71. Northern blot analysis identified a unique ~1.4kb *ER99* mRNA band in only human normal testis tissue. Interestingly, we also found that this gene also expressed in human cancer cell lines such as one human pancreatic tumour cell line, four human breast cancer cell lines (ZR-75-1, T-47D, MCF-P7 and MDA-MB-435) and three ES/PNET cell lines (SK-ES-1, RD-ES and SK-N-MC). Database analysis revealed that other research groups also found that this gene expressed in Ewing's sarcoma cell lines (Drs. D.N.T. Aryee and H. Kovar) and human gastric tumour (Dr. R. Strausberg). Furthermore, in this study, ER99 was also demonstrated to be expressed in *EWS/FLI-1* transfected NIH3T3 cells but not in NIH3T3 mock cells, suggesting that this gene was up-regulated by EWS/FLI-1. Therefore, this novel testis *ETS* gene may contribute to oncogenesis, similar to a number of other 'cancer-testis' genes.

7.4 Generation of *EWS* Gene Knockout and *EWS/Fli-1* Fusion Gene Knock-in Constructs

Homologous recombination to generate mutant chromosomal DNA in embryonic stem (ES) cells, followed by injection of the genetically altered ES cells into blastocysts, has proven to be an effective way to create mice with a designed mutation in a given gene. This technology allows us to investigate the biological functions of individual genes in mouse genome by examining the phenotypes presented by mice with a specific gene mutant.

The biological function of the *EWS* gene has been predicted from its gene structure and there have been a number of *in vitro* studies, however the normal *in vivo* functions of this gene is still unknown. One approach to determine the normal biological role served by *EWS* gene *in vivo* is to disrupt this gene and examine the consequent phenotype. Thus the generation of this mouse model has been initiated by developing a construct suitable for inserting a null mutation into this gene by homologous recombination.

This study has also demonstrated that both the murine and human EWS/FLI-1 has similar tumourigenicity. These data suggest that it is possible to develop and utilize a mouse model of Ewing's sarcoma to elucidate the aetiology of this disease. Thus a knock-in construct was developed for homologous recombination that would insert the murine *Fli-1* DNA-binding domain into the murine *EWS* genome to generate an *EWS/Fli-1* fusion gene under the control of the murine *EWS* endogenous promoter.

As mentioned in Chapter 6, the presence of murine *EWS* pseudogenes hindered the isolation of *EWS* genomic clones. Thus *Elongase* PCR was used to amplify *EWS* genomic fragments which were needed for knockout and knock-in constructs generation from 129S6/SvErTac ES cell genomic DNA. Targeting constructs were generated for both 'Knock-in' and 'Knockout' strategies using PGK-Neomycin for selection, however several attempts failed to generate targeted clones due to random integration of targeting vectors. To improve the targeting efficiency, a promoterless *IRES/Neo* has been inserted into both vectors. The electroporation and screening of these *IRES/Neo* targeting vectors are proceeding.

7.5 Future Directions

1. Identification of EWS/FLI-1 Target Genes

The identification of downstream targets of EWS/FLI-1, especially the primary target genes, will clarify the biological mechanisms of EWS/FLI-1 fusion onco-protein and will discover new therapeutic approaches to these ES/PNET tumours. The EWS/FLI-

1—transformed and KF— transformation suppressed cell models will provide valuable tools to elucidate more downstream target genes. cDNA micro-array analysis using these cells will be performed to compare the gene expression profiles (e.g. between NIH3T3 mock control vs EWS/FLI-1 transformed NIH3T3 cell line such as mEF#1 or hEF#16 vs KRAB/Fli-1 co-transfected transformed cell lines such as mEF#1K1 or hEF#16K13, and SK-N-MC mock control cells vs KRAB/Fli-1 co-transfected SK-N-MC cell lines such as SK-K16). The putative downstream target genes will be verified by Northern blot and quantitative RT-PCR. Their promoters will be examined for EWS/FLI-1 binding ability by Gel Mobility Assays and Transient Transfection Assay using correlative promoter-luciferase reporter constructs.

2. Generation and Analysis of EWS Knockout Mice

EWS gene knockout using homologous recombination will be continued. The targeted clones will be injected into blastocysts and will create mice for elucidating the functions of *EWS* gene. The *EWS* gene similar to *TLS/FUS, hTAF$_{II}$68* and *Cabeza*, sharing a consensus RNA-binding domain (RNP-CS) and belong to a new subfamily of RNP-CS-containing proteins called TET family. Thus *EWS* deficient mice will be studied as same as *TLS/FUS* deficient mice which was generated by Kuroda *et al*, 2000 and Hicks *et al*, 2000. Kuroda *et al* found that the *TLS/FUS* deficient mice are sterile with a marked increase in the number of unpaired and mis-paired chromosomal axes in premeiotic spermatocytes. Hicks *et al* found that the *TLS/FUS* deficient mice died during neonatal stage and impairment of lymphocyte development.

3. Generation of a Mouse Model for Ewing's sarcoma

As same as *EWS* gene knockout, the *EWS/FLI-1* knock-in homologous recombination experiment will be continued. Moreover, the *EWS/Fli-1* knock-in ES cells will be studied the tumourigenicity in nude mice and develop mice with *EWS/Fli-1* knock-in target cells. The knock-in mice will be crossed with tetracycline-inducible *KF* expressing mice. This can be used to study the suppression effect of KF in ES/PNET tumours *in vivo*.

3. Isolation and Characterization of Full-length of *ER99*

The identification, isolation and characterization the full-length *ER99*, will be continued using 5' RACE PCR on ZR-75-1 cell line. The full-length *ER99* cDNA of will be stably over-expressed in NIH3T3 cells to study its tumourigenicity. The expression pattern will be further studied using more variety of human cancers and using human cancer tissue array. The promoter of *ER99* will be amplified according the full-length cDNA of *ER99* and genomic sequence of Human DNA from overlapping chromosome 19 cosmids R31396, F25451 (Accession number: AC002115). The *EWS/FLI-1* binding sites will be searched on the promoter region. This will illustrate whether *EWS/FLI-1* does turn-on or *KF* does turn-off the expression of this gene. The promoter will be used to drive GFP or *LacZ* reporter genes in transgenic mice or human cancer cell lines to show the expressing cell types and localization in cells. This promoter will also be fused with a suicide gene such as a promoterless *HSV-tk* gene and transfected into human cancer cell lines such as ZR-75-1 or SK-N-MC which will be inoculated in nude mice. By administration of G418 water, a novel gene therapy method will be demonstrated on this mice assay. Finally, a mouse model with *ER99* deficiency will be generated by knockout for studying the normal function of this gene.

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Appendix 1
Lists of Media

DMEM
One pack of DMEM powder (GIBCO-BRL), add 3.7g sodium bicarbonate, and make up to 1L MQH_2O. After sterile filtered, 10% fetal bovine serum and 10mM penicillin/streptomycin are added

EMEM
One pack of Eagle's Minimal Essential medium (EMEM), added 1.5g sodium bicarbonate, and make up to 1L MQH_2O. After sterile filtered, 10% fetal bovine serum, 1.0mM sodium pyruvate, 0.1mM nonessential amino acids and 10mM penicillin/streptomycin are added

NZYM medium
10g/l NZ amine, 5g/l NaCl, 5g/l bacto-yeast extract, 2g/l $MgSO_4.7H2O$

Luria-Bertani (LB) Medium
10g bacto-tryptone, 5g bacto-yeast extract, 5g NaCl in 1 litre of MQH_2O, pH7.0 and autoclave

LB Top Agar
LB broth plus 7g agar per litre, dispense into 10 x 100mls bottles and autoclave

LB Bottom Agar
LB broth plus 15g of agar per litre, dispense into 5 x 200mls bottles and autoclave

LB/Amp Agar Plate
100mls LB bottom agar was added by 100µl Ampicillin (10mg/ml) when the temperature was at 55^0C. Five mls were poured onto 10cm plates

Terrific Broth (TB)
12g/l bacto-tryptone, 24g/l bacto-yeast extract, 4mls/l glycerol, 0.017M KH_2PO_4, 0.072 MK_2HPO_4 and autoclave

SOC medium
20g/l bacto-tryptone, 5g/l bacto-yeast extract, 0.5g/l NaCl, 20mM glucose and autoclave.

Appendix 2
Lists of Buffers and Solutions

β-gal Assay Buffer (2 x)
200mM $NaPO_4$ pH7.3, 2mM $MgCl_2$, 100mM β-Mercaptoethanol, 1.33mg/ml ONPG to 50MQH_2O

Chloroform:Isoamyl alcohol (49:1)
Mix 490mls chloroform with 10ml Isoamyl alcohol in a sterile bottle, keep in 4^0C

Cell freezing buffer
10% v/v DMSO, 90% v/v FCS

DNA gel-loading buffer
0.42% v/v bromophenol blue, 67% w/v sucrose in MQH_2O

DEPC-treated Water
999mls MQH_2O, 1ml Diethyl pyrocarbonate

DNA loading dye
0.42% w/v bromophenol blue, 67% w/v sucrose, 50% v/v glycerol

EDTA (0.5 M, pH=8)
186.12g/l EDTA, pH=8.0

Guanidinium isothiocyanate homogenization buffer (Solution D)
4M guanidinium thiocyanate, 25mM sodium citrate pH=7.0, 0.5% v/v sodium lauryl sarkosinate in DEPC-treated water. Add β-mercaptoethanol to a final 0.1M prior to use

Lower Gel Buffer (5x)
227g/l Tris, 5g/l SDS, pH=8.8

Phosphate buffered saline (PBS)
8g/l NaCl, 1.45g/l Na2HPO4, 0.2g/l KCl, 0.2g/l KH2PO4, pH=7.4

Phenol for DNA/RNA
500g phenol melted at 65^0C, equilibrated with an equal volume of 50mM Tris-HCl, pH=9.0

Protein Blocking Buffer
2% v/v FCS, 0.01% v/v TWEEN-20, 4% v/v milk powder and PBS

Protein Sample Buffer (1x)
15% v/v glycerol, 5% v/v β-Mercaptoethanol, 23% v/v SDS, 5mls 10 x upper gel buffer

RNase A (10mg/ml)
100mg RNase A, 100µl 1M Tris-HCl pH=7.5, 30µl 5M NaCl in 10mls deionised water

RNA gel-loading buffer
50% v/v glycerol, 1mM EDTA, 0.25% v/v bromophenol blue, 0.25% v/v xylene cyanol FF in MQH_2O, pH=8.0

RNA denaturing gel-running buffer
20mM MOPS, 8mM Sodium acetate, 1mM EDTA, pH=8.0

SDS Protein Running Buffer (10x)
30.3g/l Tris, 144.1g/l glycine, 10g/l SDS, pH=8.3

SDS-Polyacrylamide Gel (8%)
50mls gel solution is suitable 4 gels. 23.2mls MQH_2O, 13.3ml 30% Acrylamide mix (BIO-RAD), 13mls 5 x Lower Gel Buffer, 0.5mls 10% ammonium persulphate and 0.03ml TEMED (BIO-RAD)

SM
5.8g/l NaCl, 2g/l $MgSO_4.7H_2O$, 50mls/l of 1M Tris-Cl, 5ml/l of 2% gelatin solution

Solutions for PolyA$^+$ Extraction
Extraction buffer 0.1M NaCl, 20mM Tris, 1mM EDTA, 0.5% v/v SDS

Binding buffer 0.5M NaCl, 10mM Tris, 1mM EDTA, 0.1% v/v SDS

Wash buffer 0.15M NaCl, 10mM Tris, 1mM EDTA, 0.1% v/v SDS

Elution buffer 10mM Tris, 1mM EDTA, 0.1% v/v SDS

Solutions for Maxi Plasmid Prep
Solution I 50mM glucose, 25mM Tris-HCl pH=8.0, 10mM EDTA

Solution II 0.2N NaOH and 1% v/v SDS

Solution III 2.55M KOAc, pH=4.8

SSC (20x)
175.2g/l NaCl, 88.2g/l sodium citrate

SSPE (20x)
174g/l NaCl, 27.6g/l $NaH_2PO_4.H_2O$, 7.4g/l EDTA

Stacking Gel (5%)
20mls gel solution is suitable for 4 gels. 13.6mls MQH_2O, 3.4mls Acrylamide mix (BIO-RAD), 2.6mls 10 x Upper Gel/ Stacking Buffer, 0.2mls 10% ammonium persulphate and 0.02mls TEMED (BIO-RAD)

STT Buffer (10x)
80g/l sucrose, 50mls Triton, 50mls 1M Tris-HCl pH=8

TAE (1x)
40mM Tris-acetate, 1mM EDTA, pH=8.0

TE (1x)
10mM Tris-HCl, 1mM EDTA, pH=8.0

Trypsin/EDTA Solution
0.25% v/v Trypsin, 0.4% v/v EDTA and make up with PBS

Upper Gel/ Stacking Buffer (10x)
152g/l Tris, 10g/l SDS, pH=6.8

Appendix 3
List of Suppliers

American Tissue Culture Collection (ATCC)	Rockville, MD, USA
Amersham Pharmacia Biotech.	Buckinghamshire, UK
AMRAD	Kew, VIC, Australia
Applied Biosystems	CA, USA
Bartelt Instruments	Heidelberg W., VIC, Aust
BDH Biochemicals	Poole, UK
BIO-RAD	Richmond, CA, USA
Bochringer Mannheim Biochem	Mannheim, Germany
Clontech	Palo Alto, CA, USA
Collaborative Research	Bedford, MA, USA
CSL	Parkville, VIC, Australia
DAKO	Carpinteria, CA, USA
Difco	Michigan, USA
Falcon	Becton-Dickson, France
GIBCO-BRL	Ontario, Canada
Hoffman La Roche	Basel, Switzerland
Hoefer Scientific Instruments	San Fancisco, USA
Invitrogen	Leek, Netherlands
NEN™ Life Science Products Inc.	Boston, USA
Nunc	Roskilde, Denmark
Packard-Becker	Groningen, Netherlands
Perkin Elmer	Norwalk, Connecticut, USA
Pharmacia Biotec Inc.	Piscataway, NJ, USA
PIERCE	Rockford, Illinois, USA
Progen Industries	Rockford, IL, USA
Promega	Madison, WI, USA
Qiagen	Chatsworth, CA, USA
Roche Molecular Biochemicals	Mannheim, Germany
Santa Cruz Laboratories	Santa Cruz, CA, USA
Sigma Chemical Co.	St. Louis, MO, USA
Strategene	La Jolla, CA, USA

Appendix 4
Orientations and Sequences of Oligonucleotides

Remarks: S = sense, A = antisense, EP = *Elongase* PCR, RP = RT-PCR, P = PCR, Race PCR = RaP

Genes	Oligonucleotides	Sequence	Orientations	Use
EWS	MEWSN-AS	5'-GTTGAGAGAACGAGGAGGAAG-3'	S	EP
	MEWSN-DA	5'-GCAGTGGTCTGAGCCTGAG-3'	A	EP
	MEW1-1AB	5'-CCTTGAGCTCATGGATCCAG-3'	A	EP
	MEW2-2AB	5'-GTTGCAGAAAGCTGACTTACC-3'	A	EP
	MEWS 1S B	5'-GAAGGCGAGAAAATGGCGTCC-3'	S	P, RP, EP
	MEWSINT-2A	5'-CTACAAAAGGCCTCATACGC-3'	A	EP
	MEWSINT-2AB	5'-GTTGCAGAAAGCTGACTTACCC-3'	A	EP
	MEWSINT-3A	5'-CAAGGACTTTGTTAATCCG-3'	A	EP
	MEWSINT-3S	5'-GCCTAAGCTACAAGGTTCTC-3'	S	EP
	MEWSINT-3SB	5'-GACCTCTTCGTTTCATGTTT-3'	S	EP
	MEWS I-4S	5'-CAAATCTGGCAGAGAGGACAG-3'	S	EP
	MEWS I-7SB	5'-GAAAGGAGTCCAAGTAAGGC-3'	S	EP
	MEWSINT-7S	5'-GTGGCTTACAGATGTGACTC-3'	S	EP
	MEWS-7ISHindIII	5'-GACTGGGGAAGCTTGTTACC-3'	S	EP
	MEWS-7ISSacI	5'-GTGCTTATGTTGAGAGCTCC-3'	S	EP
	MEWS-9S	5'-GCGAGGTGGCTTCAATAAGC-3'	S	EP
	MEWS-9AHindIII	5'-GCTTATTGAAGCTTCCTCGC-3'	A	EP
	MEWS-1S	5'-GAAGGCGAGAAAATGGCGTC-3'	S	P, RP, EP
	MEWS-2A	5'-GCAGCTTGACTATAGGTACTG-3'	A	P, EP
	MEWS-3A	5'-CTGTGCATATCCTTGAGTTGG-3'	A	EP
	MEWS-5A	5'-GTGGTGACTGTAGCAGTGGTGC-3'	A	EP
	MEWS-6S	5'-CAGAGTAACTACAGCTATCCC-3'	S	P, RP, EP
	MEWS-6A	5'-TAGCTGTAGTTACTCTGTCC-3'	A	P, RP, EP
	MEWS-7S	5'-CAGCTATGGACAACAGAGTAG-3'	S	P, RP, EP
	MEWS-7A	5'-CTACTCTGTTGTCCATAGCTG-3'	A	P, RP, EP

	MEWS-8A	5'-CATGCCTCCACGATCAAATC-3'	A	P, RP, EP
	MEWS-9A	5'-GCTTATTGAAGCCACCTCGC-3'	A	P, RP, EP
	MEWS-10A	5'-CAAGATCTGGTCCTTCATCC-3'	A	RP, EP
Fli-1				
	MFli-1-6S	5'-CCCTTCTTATGACTCTGTCAGG-3'	S	P, RP
	MFli-1	5'-ACCCTTCTTATGACTCTGTC-3'	S	P, RP
	MFli-1-8A	5'-GGATCTGATAAGGATCTGGC-3'	A	P, RP
	MFli-1-9S	5'-CCAACATGAATTATGACAAGC-3'	S	P, RP
Knock-in	KIEFS	ews FLI-1		
Overlapping	5'-GCTACGGGCAGCAGAACCCTTCTTATGACTCTGTC-3'		S	P
PCR	KIEFA	fli-1 EWS		
	5'-AGTCATAAGAAGGGTTCTGCTGCCCGTAGCTGCTG-3'		A	P
Degenerative RT-PCR				
	Deg-S	5'-GGCCGGATCCCTNTGGSARTTYCTNCT-3'	S	RP
	Deg-A	5'-GGCCAAGCTTRTARTARTAYCTNAGNSG-3'	S	RP
	Where R(AG), Y(CT), S(GC), N(AGCT)			
ER99				
	ER99-3S	5'-CACCGTTTGCTCCGAACCGAGCCCG-3'	S	RP
	ER99-2A	5'-CTCAGCTTCTCGTAATTCATGCC-3'	A	RP
	ER99-3A	5'-CTCGGTTCGGAGCAAACGGTGAG-3'	A	RP
	ER99-4A	5'-GAGCTCTGGTACCGCTTCAAAG-3'	A	RP
	ER99-4S	5'-GACTCTCAGGCTCTTCCGTG-3'	S	RP
	ER99-2A-RACE	5'-GCTCAGCTTCTCGTAATTCATGCC-3'	A	RaP
RACE-3A	5'-CCGACTGCGGGCTCGGTTCGGAGCAAACGGTG-3'		A	RaP
EAT-2				
	EAT-SB	5'-GATCTGCCTTACTACCATG-3'	S	RP
	EAT-2S	5'-GGATCTGCCTTACTACCATGG-3'	S	RP
	EAT-RB	5'-GTATGAGCATCAGTCTGTTC-3'	A	RP
	EAT-2R	5'-GACGTCCACATACTCCTCATC-3'	A	RP
c-myc				
	c-mycS	5'-CTCAACGACAGCAGCTCGCC-3'	S	RP
	c-mycA	5'-GGAGACGTGGCACCTCTTGAG-3'	A	RP
GAPDH				
	GAPDH-S	5'-CTGCCACCCAGAAGACTGTGG-3'	S	RP

GAPDH-A	5'-GTCATACCAGGAAATGAGC-3'	A	RP
Stromelysin-1			
MST-1S	5'-CAGAGCTGTGGGAAGTCAATG-3'	S	RP
MST-2S	5'-CTTGTGTGCTCATCCTACC-3'	S	RP
MST-3S	5'-CATGGATGCTGCATATGAGG-3'	S	RP
MST-1A	5'-CACAGGATGCCTTCCTTGG-3'	A	RP
MST-2A	5'-CCACTGAAGAAGTAGAGAAACC-3'	A	RP
Manic Fringe			
MMF-1S	5'-CAGACTTTTGCCTGTACCAGG-3'	S	RP
MMF-2S	5'-CCTGCTACCAATGCACTGC-3'	S	RP
MMF-3S	5'-GCACTGTGGGCTACATCATC-3'	S	RP
MMF-1A	5'-GGCAGTAGCATCCATCATCC-3'	A	RP
MMF-2A	*5'-GTAGAGGAGACAATGGAGG-3'*	*A*	*RP*
KRAB			
KRAB-KOZAK			
	5'-TCCCCGCGGTTCGCCACCATGGATGCTAAGTCACTA-3'	S	P
KRAB-SB	5'-GTGGACTTCACCAGGGAGGAG-3'	S	RP
KAP-1			
KAP-1S	5'-GTGCTTCTCCAAAGACATCG-3'	S	RP
KAP-1A	5'-GGATGGCCATCTTGACATC-3'	A	RP

Appendix 5

Mouse *EWS* cDNA Accession number: X79233

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                                                    TAGGGGGAACGCGAGAAGGAGACGGACGTTGAGAGAACGAGGAGGAAGGCGAGAAA
      M  A  S  T  D  Y  S  T  Y  S  Q  A  A  A  Q  Q  G  Y  S  A  Y  T  A  Q  P  T  Q  G  Y  A   30
1     ATGGCGTCCACGGATTACAGTACCTATAGTCAAGCTGCAGCCCAGCAGGGCTACAGTGCTTACACCGCCCAGCCAACTCAAGGATATGCA
      Q  T  T  Q  A  Y  G  Q  Q  S  Y  G  T  Y  G  Q  P  T  D  V  S  Y  T  Q  A  Q  T  T  A  T   60
91    CAGACCACCCAGGCATATGGGCAACAAAGCTATGGAACCTATGGACAGCCTACTGATGTCAGCTATACTCAGGCTCAGACCACTGCCACC
      Y  G  Q  T  A  Y  A  T  S  Y  G  Q  P  P  T  G  Y  S  T  P  T  A  P  Q  A  Y  S  Q  P  V   90
181   TACGGGCAGACTGCATATGCAACTTCTTACGGACAGCCTCCCAGTGGTTATAGTACTCCAACTGCCCCCCAGGCGTACAGCCAGCCTGTG
      Q  G  Y  G  T  G  A  Y  D  S  T  T  A  T  V  T  T  T  Q  A  S  Y  A  A  Q  S  A  Y  G  T  120
271   CAGGGATATGGCACTGGGGCTTATGACAGCACCACTGCTACAGTCACCACAACGCAGGCCTCTTACGCAGCTCAGTCAGCATATGGCACC
      Q  P  A  Y  P  T  Y  G  Q  Q  P  T  A  T  A  P  T  R  P  Q  D  G  N  K  P  A  E  T  S  Q  150
361   CAGCCTGCCTACCCCACCTATGGCCAGCAGCCAACAGCCACCGCACCTACCAGACCACAGGATGGTAACAAGCCTGCTGAGACTAGCCAA
      P  Q  S  S  T  G  G  Y  N  Q  P  S  L  G  Y  G  Q  S  N  Y  S  Y  P  Q  V  P  G  S  Y  P  180
451   CCTCAATCTAGCACAGGGGGTTATAACCAACCCAGCCTAGGATATGGACAGAGTAACTACAGCTATCCCCAGGTACCTGGGAGCTACCCA
      M  Q  P  V  T  A  P  P  S  Y  P  P  T  S  Y  S  S  S  Q  P  T  S  Y  D  Q  S  S  Y  S  Q  210
541   ATGCAGCCAGTCACCGCACCTCCATCTTATCCTCCTACCAGCTACTCCTCTTCACAGCCGACTAGTTACGATCAGAGCAGTTACTCTCAG
      Q  N  T  Y  G  Q  P  S  S  Y  G  Q  Q  S  S  Y  G  Q  Q  S  S  Y  G  Q  Q  P  P  T  S  Y  240
631   CAGAACACCTATGGGCAGCCGAGCAGCTATGGACAACAGAGTAGCTATGGTCAACAAAGCAGCTATGGGCAGCAGCCTCCTACTAGTTAC
      P  P  Q  T  G  S  Y  S  Q  A  P  S  Q  Y  S  Q  Q  S  S  S  Y  G  Q  Q  S  S  F  R  Q  D  270
721   CCGCCTCAGACTGGATCCTACAGCCAGGCTCCAAGTCAATATAGCCAACAGAGCAGCAGCTACGGGCAGCAGAGTTCATTCCGACAGGAC
      H  P  S  S  M  G  V  Y  G  Q  E  S  G  G  F  S  G  P  G  E  N  R  S  L  S  G  P  D  N  R  300
810   CACCCCAGTAGCATGGGTGTTTATGGGCAGGAGTCTGGAGGATTTTCCGGACCAGGAGAGAACCGGAGCTTGAGTGGCCCTGATAACCGG
      G  R  G  R  G  G  F  D  R  G  G  M  S  R  G  G  R  G  G  G  R  G  G  L  G  A  G  E  R  G  330
901   GGCAGGGGAAGAGGGGGATTTGATCGTGGAGGCATGAGCAGAGGTGGGCGGGGAGGAGGACGCGGTGGACTGGGCGCTGGAGAGCGAGGT
      G  F  N  K  P  G  G  P  M  D  E  G  P  D  L  D  L  G  L  P  I  D  P  D  E  D  S  D  N  S  360
991   GGCTTCAATAAGCCTGGTGGACCCATGGATGAAGGACCAGATCTTGATCTAGGCCTTCCTATAGATCCCGATGAAGACTCTGACAACAGT
      A  I  Y  V  Q  G  L  N  D  N  V  T  L  D  *  L  A  D  F  F  K  Q  C  G  V  V  K  M  N  K  390
1081  GCAATTTATGTGCAAGGATTAAATGACAATGTGACTCTGGATG*TCTGGCAGACTTCTTTAAGCAGTGTGGGGTTGTCAAGATGAACAAG
      R  T  G  Q  P  M  I  H  I  Y  L  D  K  E  T  G  K  P  K  G  D  A  T  V  S  Y  E  D  P  P  420
1171  AGAACTGGACAACCCATGATCCATATCTACCTGGATAAGGAGACAGGAAAGCCTAAAGGGGACGCCACAGTGTCCTATGAAGATCCACCA
      T  A  K  A  A  V  E  W  F  D  G  K  D  F  Q  G  S  K  L  K  V  S  L  A  R  K  K  P  P  M  450
1261  ACTGCAAAGGCTGCCGTGGAATGGTTTGATGGGAAAGATTTTCAAGGAAGCAAACTTAAAGTGTCTCTTGCCCGAAAGAAGCCTCCAATG
      N  S  M  R  G  G  M  P  P  R  E  G  R  G  M  P  P  P  L  R  G  G  P  G  G  P  G  G  P  G  480
1351  AACAGCATGCGGGGAGGCATGCCACCTCGTGAGGGCAGGGGTATGCCACCACCACTTCGTGGAGGTCCTGGTGGCCCAGGAGGCCCTGGA
      G  P  M  G  R  M  G  G  R  G  G  D  R  G  G  F  P  P  R  G  P  R  G  S  R  G  N  P  S  G  510
1441  GGACCCATGGGTCGCATGGGAGGCCGTGGAGGAGACAGAGGGGGCTTCCCTCCAAGAGGGCCCCGAGGCTCCAGAGGAAACCCCTCTGGA
      G  G  N  V  Q  H  R  A  G  D  W  Q  C  P  N  P  G  C  G  N  Q  N  F  A  W  R  T  E  C  N  540
1531  GGAGGAAATGTCCAGCACCGAGCTGGAGACTGGCAGTGTCCCAATCCGGGCTGTGGAAACCAGAACTTCGCTTGGAGAACAGAATGCAAC
      Q  C  K  A  P  K  P  E  G  F  L  P  P  P  F  P  P  P  G  G  D  R  G  R  G  G  P  G  G  M  570
1621  CAGTGTAAGGCCCCTAAGCCCGAGGGCTTCCTCCCGCCACCCTTTCCACCTCCGGGTGGTGATCGTGGACGAGGTGGCCCTGGTGGCATG
      R  G  G  R  G  G  L  M  D  R  G  G  P  G  G  M  F  R  G  G  R  G  G  D  R  G  G  F  R  G  600
1711  CGAGGAGGAAGAGGAGGACTCATGGACCGTGGTGGTCCTGGAGGAATGTTCAGAGGTGGCAGAGGTGGAGACAGAGGAGGCTTCCGAGGT
      G  R  G  M  D  R  G  G  F  G  G  G  R  R  G  G  P  G  G  P  P  G  P  L  M  E  Q  M  G  G  630
1801  GGCCGTGGAATGGACCGAGGTGGCTTTGGTGGAGGAAGACGAGGTGGTCCTGGGGGGCCTCCTGGACCTTTAATGGAACAGATGGGAGGA
      R  R  G  G  R  G  G  P  G  K  M  D  K  G  E  H  R  Q  E  R  R  D  R  P  Y                655
1891  AGAAGAGGCGGACGTGGAGGACCTGGGAAAATGGATAAAGGCGAGCACCGTCAGGAACGCAGAGACCGGCCCTACTAGAGACCTGCAGAG
      CTGCATTGAGTACCAGATTTATTTTTTAAACCAGGAAATGTTTTAAATTTATAATTCCATATTTATAATGTTGGCGACAACATTATGATT
      ATTCCTTGTCTGTACTTTAGTATTTTTCACCATTTGTGGAGAAACATTAAAACAAGTTAAAT
```

Appendix 6

Mouse *Fli-1* cDNA Accession number: X59421

```
                                                    AAAGTGAAGTCACTTCCCAAAATTAGCTGAAAAAAAGTTTCATCCGGTTAACTGTCTCTTTTTC
      GATCCGCTACAACAACAAACGTGCACAGGGGAGCGAGGGCAGGGCGCTCGCAGGGGGCACTCAGAGAGGGCCCAGGGCGCCAAAGAGGCC
1     GCGCCGGGCTAATCTGAAGGGGCTACGAGGTCAGGCTGTAACCGGGTCAATGTGTGGAATATTGGGGGGCTCGGCTGCAGACTTGGCCAA
       M  D  G  T  I  K  E  A  L  S  V  V  S  D  D  Q  S  L  F  D  S  A  Y  G  A  A  A  H  L  P   30
91    ATGGACGGGACTATTAAGGAGGCTCTGTCTGTGGTGAGTGACGATCAGTCCTTTTTGATTCAGCATACGGAGCGGCAGCCCATCTCCCC
       K  A  D  M  T  A  S  G  S  P  D  Y  G  Q  P  H  K  I  N  P  L  P  P  Q  Q  E  W  I  N  Q   60
181   AAGGCAGATATGACTGCTTCGGGGAGTCCTGACTACGGGCAGCCCCACAAAATCAACCCCCTGCCACCGCAGCAGGAGTGGATCAACCAG
       P  V  R  V  N  V  K  R  E  Y  D  H  M  N  G  S  R  E  S  P  V  D  C  S  V  S  K  C  N  K   90
271   CCAGTGAGAGTCAATGTCAAGCGGGAGTATGACCACATGAATGGATCCAGGGAGTCTCCGGTGGACTGCAGTGTCAGCAAATGTAACAAG
       L  V  G  G  G  E  A  N  P  M  N  Y  N  S  Y  M  D  E  K  N  G  P  P  P  P  N  M  T  T  N  120
361   CTGGTGGGCGGAGGCGAAGCCAACCCCATGAACTATAATAGCTACATGGATGAGAAGAACGGCCCCCCTCCTCCCAACATGACCACCAAC
       E  R  R  V  I  V  P  A  D  P  T  L  W  T  Q  E  H  V  R  Q  W  L  E  W  A  I  K  E  Y  G   150
451   GAACGGAGAGTCATTGTGCCTGCAGACCCCACACTGTGGACACAGGAGCACGTTCGACAGTGGCTGGAGTGGGCTATAAAGGAATACGGA
       L  M  E  I  D  T  S  F  F  Q  N  M  D  G  K  E  L  C  K  M  N  K  E  D  F  L  R  A  T  S   180
541   TTGATGGAGATTGACACTTCCTTCTTCCAGAACATGGATGGCAAGGAATTGTGTAAAATGAACAAGGAGGACTTCCTCCGAGCCACCTCC
       A  Y  N  T  E  V  L  L  S  H  L  S  Y  L  R  E  S  S  L  L  A  Y  N  T  T  S  H  T  D  Q   210
631   GCCTACAACACAGAAGTGCTGTTGTCGCACCTCAGTTACCTCAGGGAAAGTTCACTGCTGGCCTATAACACAACCTCCCATACAGACCAG
       S  S  R  L  N  V  K  E  D  P  S  Y  D  S  V  R  R  G  A  W  N  N  N  N  M  N  S  G  L  N  K   240
721   TCCTCACGACTGAATGTCAAGGAAGACCCTTCTTATGACTCTGTCAGGAGAGGAGCATGGAACAATAATATGAACTCTGGCCTCAACAAA
       S  P  L  L  G  G  S  Q  T  M  G  K  N  T  E  Q  R  P  Q  P  D  P  Y  Q  I  L  G  P  T  S   270
810   AGTCCTCTCCTTGGAGGATCACAGACCATGGGCAAGAACACTGAGCAGCGGCCCCAGCCAGATCCTTATCAGATCCTGGGGCCAACCAGC
       S  R  L  A  N  P  G  S  G  Q  I  Q  L  W  Q  F  L  L  E  L  L  S  D  S  A  N  A  S  C  I   300
901   AGCCGCCTAGCAAACCCTGGGAGTGGGCAGATCCAGCTGTGGCAGTTTCTCCTGGAACTACTGTCCGACAGCGCCAACGCCAGCTGTATC
       T  W  E  G  T  N  G  E  F  K  M  T  D  P  D  E  V  A  R  R  W  G  E  R  K  S  K  P  N  M   330
991   ACCTGGGAGGGGACCAACGGGGAGTTCAAAATGACGGACCCTGATGAGGTGGCCAGGCGCTGGGGAGAGCGGAAGAGCAAGCCCAACATG
       N  Y  D  K  L  S  R  A  L  R  Y  Y  Y  D  K  N  I  M  T  K  V  H  G  K  R  Y  A  Y  K  F   360
1081  AATTATGACAAGCTGAGCCGGGCCCTCCGATACTACTATGACAAAAACATTATGACCAAAGTGCATGGCAAAAGGTATGCCTACAAGTTT
       D  F  H  G  I  A  Q  A  L  Q  P  H  P  T  E  T  S  M  Y  K  Y  P  S  D  I  S  Y  M  P  S   390
1171  GACTTCCATGGCATTGCCCAGGCCCTGCAGCCACATCCAACAGAGACATCCATGTACAAGTATCCCTCTGATATCTCCTACATGCCTTCC
       Y  H  A  H  Q  Q  K  V  N  F  V  P  S  H  P  S  S  M  P  V  T  S  S  S  F  F  G  A  A  S   420
1261  TACCATGCCCATCAACAGAAGGTGAACTTTGTCCCGTCTCACCCATCCTCCATGCCTGTCACCTCCTCCAGCTTCTTTGGAGCAGCATCA
       Q  Y  W  T  S  P  T  A  G  I  Y  P  N  P  S  V  P  R  H  P  N  T  H  V  P  S  H  L  G  S   450
1351  CAATACTGGACCTCCCCCACTGCTGGGATCTATCCAAACCCCAGTGTCCCCCGCCATCCTAACACCCACGTGCCTTCACACTTAGGCAGC
       Y  Y                                                                                        452
1441  TACTACTAGAACTAACACCAGTTGGCCTTCTGGCTGAAGTTCCAGCTCTCACTTTACTGGATACTCTGGACTCTAAAAGGCACAGTAGCC
      TTGAAGAGATAAGAAAACTGGATGTTCTTTCTTTTGGATACAACC
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Appendix 7

Murine *EWS/Fli-1* Fusion Gene

EWS

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                                                                GGAAGGCGAGAAA
     M  A  S  T  D  Y  S  T  Y  S  Q  A  A  A  Q  Q  G  Y  S  A  Y  T  A  Q  P  T  Q  G  Y  A   30
1    ATGGCGTCCACGGATTACAGTACCTATAGTCAAGCTGCAGCCCAGCAGGGCTACAGTGCTTACACCGCCCAGCCAACTCAAGGATATGCA
     Q  T  T  Q  A  Y  G  Q  Q  S  Y  G  T  Y  G  Q  P  T  D  V  S  Y  T  Q  A  Q  T  T  A  T   60
91   CAGACCACCCAGGCATATGGGCAACAAAGCTATGGAACCTATGGACAGCCTACTGATGTCAGCTATACTCAGGCTCAGACCACTGCCACC
     Y  G  Q  T  A  Y  A  T  S  Y  G  Q  P  P  T  G  Y  S  T  P  T  A  P  Q  A  Y  S  Q  P  V   90
181  TACGGGCAGACTGCATATGCAACTTCTTACGGACAGCCTCCCACTGGTTATAGTACTCCAACTGCCCCCCAGGCGTACAGCCAGCCTGTG
     Q  G  Y  G  T  G  A  Y  D  S  T  T  A  T  V  T  T  T  Q  A  S  Y  A  A  Q  S  A  Y  G  T  120
271  CAGGGATATGGCACTGGGGCTTATGACAGCACCACTGCTACAGTCACCACAACGCAGGCCTCTTACGCAGCTCAGTCAGCATATGGCACC
     Q  P  A  Y  P  T  Y  G  Q  Q  P  T  A  T  A  P  T  R  P  Q  D  G  N  K  P  A  E  T  S  Q  150
361  CAGCCTGCCTACCCCACCTATGGCCAGCAGCCAACAGCCACCGCACCTACCAGACCACAGGATGGTAACAAGCCTGCTGAGACTAGCCAA
     P  Q  S  S  T  G  G  Y  N  Q  P  S  L  G  Y  G  Q  S  N  Y  S  Y  P  Q  V  P  G  S  Y  P  180
451  CCTCAATCTAGCACAGGGGGTTATAACCAACCCAGCCTAGGATATGGACAGAGTAACTACAGCTATCCCCAGGTACCTGGGAGCTACCCA
     M  Q  P  V  T  A  P  P  S  Y  P  P  T  S  Y  S  S  S  Q  P  T  S  Y  D  Q  S  S  Y  S  Q  210
541  ATGCAGCCAGTCACCGCACCTCCATCTTATCCTCCTACCAGCTACTCCTCTTCACAGCCGACTAGTTACGATCAGAGCAGTTACTCTCAG
     Q  N  T  Y  G  Q  P  S  S  Y  G  Q  Q  S  S  Y  G  Q  Q  S  S  Y  G  Q  Q  P  P  T  S  Y  240
631  CAGAACACCTATGGGCAGCCGAGCAGCTATGGACAACAGAGTAGCTATGGTCAACAAAGCAGCTATGGGCAGCAGCCTCCTACTAGTTAC
     P  P  Q  T  G  S  Y  S  Q  A  P  S  Q  Y  S  Q  Q  S  S  S  Y  G  Q  Q  N  P  S  Y  D  S  270
721  CCGCCTCAGACTGGATCCTACAGCCAGGCTCCAAGTCAATATAGCCAACAGAGCAGCAGCTACGGGCAGCAGAACCCTTCTTATGACTCT
     V  R  R  G  A  W  N  N  N  M  N  S  G  L  N  K  S  P  L  L  G  G  S  Q  T  M  G  K  N  T  300
810  GTCAGGAGAGGAGCATGGAACAATAATATGAACTCTGGCCTCAACAAAAGTCCTCTCCTTGGAGGATCACAGACCATGGGCAAGAACACT
     E  Q  R  P  Q  P  D  P  Y  Q  I  L  G  P  T  S  S  R  L  A  N  P  G  S  G  Q  I  Q  L  W  330
901  GAGCAGCGGCCCCAGCCAGATCCTTATCAGATCCTGGGGCCAACCAGCAGCCGCCTAGCAAACCCTGGGAGTGGGCAGATCCAGCTGTGG
     Q  F  L  L  E  L  L  S  D  S  A  N  A  S  C  I  T  W  E  G  T  N  G  E  F  K  M  T  D  P  360
991  CAGTTTCTCCTGGAACTACTGTCCGACAGCGCCAACGCCAGCTGTATCACCTGGGAGGGGACCAACGGGGAGTTCAAAATGACGGACCCT
     D  E  V  A  R  R  W  G  E  R  K  S  K  P  N  M  N  Y  D  K  L  S  R  A  L  R  Y  Y  Y  D  390
1081 GATGAGGTGGCCAGGCGCTGGGGAGAGCGGAAGAGCAAGCCCAACATGAATTATGACAAGCTGAGCCGGGCCCTCCGATACTACTATGAC
     K  N  I  M  T  K  V  H  G  K  R  Y  A  Y  K  F  D  F  H  G  I  A  Q  A  L  Q  P  H  P  T  420
1171 AAAAACATTATGACCAAAGTGCATGGCAAAAGGTATGCCTACAAGTTTGACTTCCATGGCATTGCCCAGGCCCTGCAGCCACATCCAACA
     E  T  S  M  Y  K  Y  P  S  D  I  S  Y  M  P  S  Y  H  A  H  Q  Q  K  V  N  F  V  P  S  H  450
1261 GAGACATCCATGTACAAGTATCCCTCTGATATCTCCTACATGCCTTCCTACCATGCCCATCAACAGAAGGTGAACTTTGTCCCGTCTCAC
     P  S  S  M  P  V  T  S  S  S  F  P  G  A  A  S  Q  Y  W  T  S  P  T  A  G  I  Y  P  N  P  480
1351 CCATCCTCCATGCCTGTCACCTCCTCCAGCTTCTTTGGAGCAGCATCACAATACTGGACCTCCCCCACTGCTGGGATCTATCCAAACCCC
     S  V  P  R  H  P  N  T  H  V  P  S  H  L  G  S  Y  Y                                      498
1441 AGTGTCCCCCGCCATCCTAACACCCACGTGCCTTCACACTTAGGCAGCTACTACTAGAACTAACACCAGTTGGCCTTCTGGCTGAAGTTC
     CAGCTCTCACTTTACTGGATACTCTGGACTCTAAAAGGCACAGTAGCCTTGAAGAGATAAGAAAACTGGATGTTCTTTCTTTTGGATAGA
     ACC
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Fli-1

Appendix 8

Human *EWS* cDNA Access number: XM 009902

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                                                        AGAGGGAAAGCGAGAGGGAGACGGACGTTGAGAGAACGAGGAGGAACGAGAGAAA
     M  A  S  T  D  Y  S  T  Y  S  Q  A  A  A  Q  Q  G  Y  S  A  Y  T  A  Q  P  T  Q  G  Y  A    30
1    ATGGCGTCCACGGATTACAGTACCTATAGCCAAGCTGCAGCGCAGCAGGGCTACAGTGCTTACACCGCCCAGCCCACTCAAGGATATGCA
     Q  T  T  Q  A  Y  G  Q  Q  S  Y  G  T  Y  G  Q  P  T  D  V  S  Y  T  Q  A  Q  T  T  A  T    60
91   CAGACCACCCAGGCATATGGGCAACAAAGCTATGGAACCTATGGACAGCCCACTGATGTCAGCTATACCCAGGCTCAGACCACTGCAACC
     Y  G  Q  T  A  Y  A  T  S  Y  G  Q  P  P  T  G  Y  T  T  P  T  A  P  Q  A  Y  S  Q  P  V    90
181  TATGGGCAGACCGCCTATGCAACTTCTTATGGACAGCCTCCCACTGGTTATACTACTCCAACTGCCCCCCAGGCATACAGCCAGCCTGTC
     Q  G  Y  G  T  G  A  Y  D  T  T  T  A  T  V  T  T  T  Q  A  S  Y  A  A  Q  S  A  Y  G  T   120
271  CAGGGGTATGGCACTGGTGCTTATGATACCACCACTGCTACAGTCACCACCACCCAGGCCTCCTATGCAGCTCAGTCTGCATATGGCACT
     Q  P  A  Y  P  A  Y  G  Q  Q  P  A  A  T  A  P  T  R  P  Q  D  G  N  K  P  T  E  T  S  Q   150
361  CAGCCTGCTTATCCAGCCTATGGGCAGCAGCCAGCAGCCACTGCACCTACAAGACCGCAGGATGGAAACAAGCCCACTGAGACTAGTCAA
     P  Q  S  S  T  G  G  Y  N  Q  P  S  L  G  Y  G  Q  S  N  Y  S  Y  P  Q  V  P  G  S  Y  P   180
451  CCTCAATCTAGCACAGGGGGTTACAACCAGCCCAGCCTAGGATATGGACAGAGTAACTACAGTTATCCCCAGGTACCTGGGAGCTACCCC
     M  Q  P  V  T  A  P  P  S  Y  P  P  T  S  Y  S  S  T  Q  P  T  S  Y  D  Q  S  S  Y  S  Q   210
541  ATGCAGCCAGTCACTGCACCTCCATCCTACCCTCCTACCAGCTATTCCTCTACACAGCCGACTAGTTATGATCAGAGCAGTTACTCTCAG
     Q  N  T  Y  G  Q  P  S  S  Y  G  Q  Q  S  S  Y  G  Q  Q  S  S  Y  G  Q  Q  P  P  T  S  Y   240
631  CAGAACACCTATGGGCAACCGAGCAGCTATGGACAGCAGAGTAGCTATGGTCAACAAAGCAGCTATGGGCAGCAGCCTCCCACTAGTTAC
     P  P  Q  T  G  S  Y  S  Q  A  P  S  Q  Y  S  Q  Q  S  S  S  Y  G  Q  Q  R  P  M  D  E  G   270
721  CCACCCCAAACTGGATCCTACAGCCAAGCTCCAAGTCAATATAGCCAACAGAGCAGCAGCTACGGGCAGCAGAGACCCATGGATGAAGGA
     P  D  L  D  L  G  P  P  V  D  P  D  E  D  S  D  N  S  A  I  Y  V  Q  G  L  N  D  S  V  T   300
810  CCAGATCTTGATCTAGGCCCACCTGTAGATCCAGATGAAGACTCTGACAACAGTGCAATTTATGTACAAGGATTAAATGACAGTGTGACT
     L  D  D  L  A  D  F  F  K  Q  C  G  V  V  K  M  N  K  R  T  G  Q  P  M  I  H  I  Y  L  D   330
901  CTAGATGATCTGGCAGACTTCTTTAAGCAGTGTGGGGTTGTTAAGATGAACAAGAGAACTGGTCAACCCATGATCCACATCTACCTGGAC
     K  E  T  G  K  P  K  G  D  A  T  V  S  Y  E  D  P  P  T  A  K  A  A  V  E  W  F  D  G  K   360
991  AAGGAAACAGGAAAGCCCAAAGGCGATGCCACAGTGTCCTATGAAGACCCACCCACTGCCAAGGCTGCCGTGGAATGGTTTGATGGGAAA
     D  F  Q  G  S  K  L  K  V  S  L  A  R  K  K  P  P  M  N  S  M  R  G  G  L  P  P  R  E  G   390
1081 GATTTTCAAGGGAGCAAACTTAAACTCTCCCTTGCTCGGAAGAAGCCTCCAATGAACAGTATGCGGGGTGGTCTGCCACCCCGTGAGGGC
     R  G  M  P  P  P  L  R  G  G  P  G  G  P  G  G  P  M  G  R  M  G  G  R  G  G  D   420
1171 AGAGGCATGCCACCACCACTCCGTGGAGGTCCAGGAGGCCCAGGAGGTCCTGGGGGACCCATGGGTCGCATGGGAGGCCGTGGAGGAGAT
     R  G  G  F  P  P  R  G  P  R  G  S  R  G  N  P  S  G  G  G  N  V  Q  H  R  A  G  D  W  Q   450
1261 AGAGGAGGCTTCCCTCCAAGAGGACCCCGGGGTTCCCGAGGGAACCCCTCTGGAGGAGGAAACGTCCAGCACCGAGCTGGAGACTGGCAG
     C  P  N  P  G  C  G  N  Q  N  F  A  W  R  T  E  C  N  Q  C  K  A  P  K  P  E  G  F  L  P   480
1351 TGTCCCAATCCGGGTTGTGGAAACCAGAACTTCGCCTGGAGAACAGAGTGCAACCAGTGTAAGGCCCCAAAGCCTGAAGGCTTCCTCCCG
     P  P  F  P  P  P  G  G  D  R  G  R  G  G  P  G  G  M  R  G  G  P  G  G  L  M  D  R  G  G   510
1441 CCACCCTTTCCGCCCCCGGGTGGTGATCGTGGCAGAGGTGGCCCTGGTGGCATGCGGGGAGGAAGAGGTGGCCTCATGGATCGTGGTGGT
     P  G  G  M  F  R  G  G  R  G  G  D  R  G  G  F  R  G  G  R  G  M  D  R  G  G  F  G  G  G   540
1531 CCCGGTGGAATGTTCAGAGGTGGCCGTGGTGGAGACAGAGGTGGCTTCCGTGGTGGCCGGGGCATGGACCGAGGTGGCTTTGGTGGAGGA
     R  R  G  G  P  G  G  P  P  G  P  L  M  E  Q  M  G  G  R  R  G  G  R  G  G  P  G  K  M  D   570
1621 AGACGAGGTGGCCCTGGGGGGCCCCCTGGACCTTTGATGGAACAGATGGGAGGAAGAAGAGGAGGACGTGGAGGACCTGGAAAAATGGAT
     K  G  E  H  R  Q  E  R  R  D  R  P  Y                                                      583
1711 AAAGGCGAGCACCGTCAGGAGCGCAGAGATCGGCCCTACTAGATGCAGAGACCCCGCAGAGCTGCATTGACTACCAGATTTATTTTTTAA
     ACCAGAAAATGTTTTAAATTTATAATTCCATATTTATAATGTTGGCCACAACATTATGATTATTCCTTGTCTGTACTTTAGTATTTTTCA
     CCATTTGTGAAGAAACATTAAAACAAGTTAAATGGTAGTGTGCGGAGTTTTTTTTTCTTCCTTCTTTTAAAAATGGTTGTTTAAGACTTT
     AACAATGGGAACCCCTTGTGAGCATGCTCAGTATCATTGTGGAGAACCAAGAGGGCCTCTTAACTGTAACAATGTTCATGGTTGTGATGT
     TTTTTTTTTTTTTTTAAATAAAATTCCAAATGTTTATAA
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Appendix 9

Human *FLI-1* Accession number: NM 002017

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          GAATTCCCAAACGTGCACAGGGGAGTGAGGGCAGGGCGCTCGCAGGGGGCACGCAGGGAGGGCCCAGGGCGCCAGGGAGGCC
1     GCGCCGGGCTAATCCGAAGGGGCTGCGAGGTCAGGCTGTAACCGGGTCAATGTGTGGAATATTGGGGGGCTCGGCTGCAGACTTGGCCAA
       M  D  G  T  I  K  E  A  L  S  V  V  S  D  D  Q  S  L  F  D  S  A  Y  G  A  A  A  H  L  P   30
91    ATGGACGGGACTATTAAGGAGGCTCTGTCGGTGGTGAGCGACGACCAGTCCCTCTTTGACTCAGCGTACGGAGCGGCAGCCCATCTCCCC
       K  A  D  M  T  A  S  G  S  P  D  Y  G  Q  P  H  K  I  N  P  L  P  P  Q  Q  E  W  I  N  Q   60
181   AAGGCCGACATGACTGCCTCGGGGAGTCCTGACTACGGGCAGCCCCACAAGATCAACCCCCTCCCACCACAGCAGGAGTGGATCAATCAG
       P  V  R  V  N  V  K  R  E  Y  D  H  M  N  G  S  R  E  S  P  V  D  C  S  V  S  K  C  S  K   90
271   CCAGTGAGGGTCAACGTCAAGCGGGAGTATGACCACATGAATGGATCCAGGGAGTCTCCGGTGGACTGCAGCGTTAGCAAATCCAGCAAG
       L  V  G  G  G  E  S  N  P  M  N  Y  N  S  Y  M  D  E  K  N  G  P  P  P  P  N  M  T  T  N  120
361   CTGGTGGGCGGAGGCGAGTCCAACCCCATGAACTACAACAGCTATATGGACGAGAAGAATGGCCCCCCTCCTCCCAACATGACCACCAAC
       E  R  R  V  I  V  P  A  D  P  T  L  W  T  Q  E  H  V  -  Q  W  L  E  W  A  I  K  E  Y  S  150
451   GAGAGGAGAGTCATCGTCCCCGCAGACCCCACACTGTGGACACAGGAGCATGTGA-GCAATGGCTGGAGTGGGCCATAAAGGAGTACAGC
       L  M  E  I  D  T  S  F  F  Q  N  M  D  G  K  E  L  C  K  M  N  K  E  D  F  L  R  A  T  T  180
541   TTGATGGAGATCGACACATCCTTTTTCCAGAACATGGATGGCAAGGAACTGTGTAAAATGAACAAGGAGGACTTCCTCCGCGCCACCACC
       L  Y  N  T  E  V  L  L  S  H  L  S  Y  L  R  E  S  S  L  L  A  Y  N  T  T  S  H  T  D  Q  210
631   CTCTACAACACGGAAGTGCTGTTGTCACACCTCAGTTACCTCAGGGAAAGTTCACTGCTGGCCTATAATACAACCTCCCACACCGACCAA
       S  S  R  L  S  V  K  E  D  P  S  Y  D  S  V  R  R  G  A  W  G  N  N  M  N  S  G  L  N  K  240
721   TCCTCACGATTGAGTGTCAAAGAAGACCCTTCTTATGACTCAGTCAGAAG-GAGCTTGGGGCAATAACATGAATTCTGGCCTCAACAAA
       S  P  P  L  G  G  A  Q  T  I  S  K  N  T  E  Q  R  P  Q  P  D  P  Y  Q  I  L  G  P  T  S  270
810   AGTCCTCCCCTTGGAGGGGCACAAACGATCAGTAAGAATACAGAGCAACGGCCCCAGCCAGATCCGTATCAGATCCTGGGCCCGACCAGC
       S  R  L  A  N  P  G  S  G  Q  I  Q  L  W  Q  F  L  L  E  L  L  S  D  S  A  N  A  S  C  I  300
901   AGTCGCCTAGCCAACCCTGGAAGCGGGCAGATCCAGCTGTGGCAATTCCTCCTGGAGCTGCTCTCCGACAGCGCCAACGCCAGCTGTATC
       T  W  E  G  T  N  G  E  F  K  M  T  D  P  D  E  V  A  R  R  W  G  E  R  K  S  K  P  N  M  330
991   ACCTGGGAGGGGACCAACGGGGAGTTCAAAATGACGGACCCCGATGAGGTGGCCAGGCGCTGGGGCGAGCGGAAAAGCAAGCCCAACATG
       N  Y  D  K  L  S  R  A  L  R  Y  Y  Y  D  K  N  I  M  T  K  V  H  G  K  R  Y  A  Y  K  F  360
1081  AATTACGACAAGCTGAGCCGGGCCCTCCGTTATTACTATGATAAAAACATTATGACCAAAGTGCACGGCAAAAGATATGCTTACAAATTT
       D  F  H  G  I  A  Q  A  L  Q  P  H  P  T  E  S  S  M  Y  K  Y  P  S  D  I  S  Y  M  P  S  390
1171  GACTTCCACGGCATTGCCCAGGCTCTGCAGCCACATCCGACCGAGTCGTCCATGTACAAGTACCCTTCTGACATCTCCTACATGCCTTCC
       Y  H  A  H  Q  Q  K  V  N  F  V  P  P  H  P  S  S  M  P  V  T  S  S  S  F  F  G  A  A  S  420
1261  TACCATGCCCACCAGCAGAAGGTGAACTTTGTCCCTCCCCATCCATCCTCCATGCCTGTCACTTCCTCCAGCTTCTTTGGAGCCGCATCA
       Q  Y  W  T  S  P  T  G  G  I  Y  P  N  P  N  V  P  R  H  P  N  T  H  V  P  S  H  L  G  S  450
1351  CAATACTGGACCTCCCCCACGGGGGGAATCTACCCCAACCCCAACGTCCCCCGCCATCCTAACACCCACGTGCCTTCACACTTAGGCAGC
       Y  Y                                                                                      452
1441  TACTACTAGAAGCTTCTTCTAGCTGAAGCCCATCCTGCACACTTACTGGATGCTTTGGACTCAACAGGACATATGTGGCCTTGAAGGGAA
      GACAAAACTGGATGTTCTTTCTTGTTGGATAGAACCTTTGTATTTGTTCTTTAAAAACATTTTTTTTAATGTTGGTAACTTTTGCTTCCT
      CTACCTGAACAAAGAGATGAATAATTCCATGGGCCAGTATGCCAGTTTGAATTCTCAGTCTCCTAGCATCTTGTGAGTTGCATATTAAGA
      TTACTGGAATGGTTAAGTCATGGTTCTGAGAAAGAAGCTGTACGTTTTCTTTATGTTTTTATGACCAAAGCAGTTTCTTGTCAATACACG
      GGGTTCAGTATGACACAGAATCATGGACTTAACCCGTCATGTTCTGGTTTGAGATTTAGTGACAAATAGAGGTGGCAAGCTTATAATCTA
      ATTTTAGGAGGACCAAATTCAGCGGATGGCAACTGGAACATTGATTGTAAGGCCAGTGAAGTTTTCACCCAACTGGAATTTGATGGAAAG
      AAGGTTTGTGTGTTTAAGACGCCAAGGGCATTGCAGAATCCCTCTCAGTGGACAGTATGCACTCAGCTGACCACTCTCTCTAGAAATAGT
      CAAGATATGAACTAAGAAATTTTAATGCAAATACATACATTCCTGAAAGACGGGGAATTAAATTACTAATTTTTTTTTTTTAAATGATGA
      CAGTGGTCCCAGAACTTGGAAAAGTTGTAGGGATTTCTAAACTCAAGC-GATTCGCAAGTGCTGTGCGCTTGTCAGACCATCAGACCAGG
      GCCAACCAATCAGAAGGCAACTTACTGTATAAATTATGCAGAGTTATTTTCCTATATCTCACAGTATTAAAAAATAAATAATTAAAAATT
      AAGAATAAATAAACGAGTTGACCTCGGTCACAAAAGCAGTTTTACTATCGAATCAATCGCTGTTATTTTTTTTTAATGTAATTTGTACAT
      CTTTTTTCAATCTGTACATTTGGGCTGTCTTGTATGTTTTTATGCTCCTTTTTAAAAAGCATAATATGCCTATAGCTGAAAAGGAAACAG
      GGCTGTTTAAGTCACTGACTTATGAGAAAGCAAAGCACTGGTACAGTTATTTAACAGGCATACACAAGCAGGGAAAAGATAATCCATTTA
      GATCTTTAATGCTTTGGAAATGCGTGTAACAGTACTGCAATAATCACAGCTCTGGGAAAAACAACGAAACTTTCCCTTGTGGAGAGGAGG
      GATTTTCCTGCTCTATATAAGCAACATATTTTTAGACATTAAAATATATATAATTTTGCAGGTAATTGTTGACTTTTTTAACTATATTAA
      GTGTTAAGCTGACAACTGTCAAAGAAGACCATGTTGTAAAATAATTTGACTAAATAAATGGTTCCTTCTCTCAAAAAAAAAAAAA
```

Appendix 10

Human Type 1 *EWS/FLI-1* cDNA

```
                                                                  AGAGGGAAAGCGAGAGGGAGACGGACGTTGAGAGAACGAGGAGGAAGGAGAGAAA
     M  A  S  T  D  Y  S  T  Y  S  Q  A  A  A  Q  Q  G  Y  S  A  Y  T  A  Q  P  T  Q  G  Y  A    30
1    ATGGCGTCCACGGATTACAGTACCTATAGCCAAGCTGCAGCGCAGCAGGGCTACAGTGCTTACACCGCCCAGCCCACTCAAGGATATGCA
     Q  T  T  Q  A  Y  G  Q  Q  S  Y  G  T  Y  G  Q  P  T  D  V  S  Y  T  Q  A  Q  T  T  A  T    60
91   CAGACCACCCAGGCATATGGGCAACAAAGCTATGGAACCTATGGACAGCCCACTGATGTCAGCTATACCCAGGCTCAGACCACTGCAACC
     Y  G  Q  T  A  Y  A  T  S  Y  G  Q  P  P  T  G  Y  T  T  P  T  A  P  Q  A  Y  S  Q  P  V    90
181  TATGGGCAGACCGCCTATGCAACTTCTTATGGACAGCCTCCCACTGGTTATACTACTCCAACTGCCCCCCAGGCATACAGCCAGCCTGTC
     Q  G  Y  G  T  G  A  Y  D  T  T  T  A  T  V  T  T  T  Q  A  S  Y  A  A  Q  G  A  Y  G  T   120
271  CAGGGGTATGGCACTGGTGCTTATGATACCACCACTGCTACAGTCACCACCACCCAGGCCTCCTATGCAGCTCAGTCTGCATATGGCACT
     Q  P  A  Y  P  A  Y  G  Q  Q  P  A  A  T  A  P  T  R  P  Q  D  G  N  K  P  T  E  T  S  Q   150
361  CAGCCTGCTTATCCAGCCTATGGGCAGCAGCCAGCAGCCACTGCACCTACAAGACCGCAGGATGGAAACAAGCCCACTGAGACTAGTCAA
     P  Q  S  S  T  G  G  Y  N  Q  P  S  L  G  Y  G  Q  S  N  Y  S  Y  P  Q  V  P  G  S  Y  P   180
451  CCTCAATCTAGCACAGGGGGTTACAACCAGCCCAGCCTAGGATATGGACAGAGTAACTACAGTTATCCCCAGGTACCTGGGAGCTACCCC
     M  P  P  V  T  A  P  P  S  Y  P  P  T  S  Y  S  S  T  Q  P  T  S  Y  D  Q  S  S  Y  S  Q   210
541  ATGCCAGCCAGTCACTGCACCTCCATCCTACCCTCCTACCAGCTATTCCTCTACACAGCCGACTAGTTATGATCAGAGCAGTTACTCTCAG
     Q  N  T  Y  G  Q  P  S  S  Y  G  Q  Q  S  S  Y  G  Q  Q  S  S  Y  G  Q  Q  N  E  S  Y  D   240
631  CAGAACACCTATGGGCAACCGAGCAGCTATGGACAGCAGAGTAGCTATGGTCAACAAAGCAGCTATGGGCAGCAGAACCCTTCTTATGAC
     S  V  R  R  G  A  W  G  N  N  M  N  S  G  L  N  K  S  P  P  L  G  G  A  Q  T  I  S  K  N   270
721  TCAGTCAGAAGAGGAGCTTGGGGCAATAACATGAATTCTGGCCTCAACAAAAGTCCTCCCCTTGGAGGGGCACAAACGATCAGTAAGAAT
     T  E  Q  R  P  Q  P  D  P  Y  Q  I  L  G  P  T  S  S  R  L  A  N  P  G  S  G  Q  I  Q  L   300
810  ACAGAGCAACGGCCCCAGCCAGATCCGTATCAGATCCTGGGCCCGACCAGCAGTCGCCTAGCCAACCCTGGAAGCGGGCAGATCCAGCTG
     W  Q  P  L  L  E  L  L  S  D  S  A  N  A  S  C  I  T  W  E  G  T  N  G  E  F  K  M  T  D   330
901  TGGCAATTCCTCCTGGAGCTGCTCTCCGACAGCGCCAACGCCAGCTGTATCACCTGGGAGGGGACCAACGGGGAGTTCAAAATGACGGAC
     P  D  E  V  A  R  R  W  G  E  R  K  S  K  P  N  M  N  Y  D  K  L  S  R  A  L  R  Y  Y  Y   360
991  CCCGATGAGGTGGCCAGGCGCTGGGGCGAGCGGAAAAGCAAGCCCAACATGAATTACGACAAGCTGAGCCGGGCCCTCCGTTATTACTAT
     D  K  N  I  M  T  K  V  H  G  K  R  Y  A  Y  K  F  D  F  H  G  I  A  Q  A  L  Q  P  H  P   390
1081 GATAAAAACATTATGACCAAAGTGCACGGCAAAAGATATGCTTACAAATTTGACTTCCACGGCATTGCCCAGGCTCTGCAGCCACATCCG
     T  E  S  S  M  Y  K  Y  P  S  D  I  S  Y  M  P  S  Y  H  A  H  Q  G  K  V  N  F  V  P  P   420
1171 ACCGAGTCGTCCATGTACAAGTACCCTTCTGACATCTCCTACATGCCTTCCTACCATGCCCACCAGCAGAAGGTGAACTTTGTCCCTCCC
     H  P  S  S  M  P  V  T  S  S  S  F  F  G  A  A  S  Q  Y  W  T  S  P  T  G  G  I  Y  P  N   450
1261 CATCCATCCTCCATGCCTGTCACTTCCTCCAGCTTCTTTGGAGCCGCATCACAATACTGGACCTCCCCCACGGGGGGAATCTACCCCAAC
     P  N  V  P  R  H  P  N  T  H  V  P  S  H  L  G  S  Y  Y                                    469
1351 CCCAACGTCCCCCGCCATCCTAACACCCACGTGCCTTCACACTTAGGCAGCTACTACTAGAAGCTT
```

Appendix 11

Human KRAB/FLI-1 Fusion Gene

```
                                                                  CTTGGTTCGCC
      M  D  A  K  S  L  T  A  W  S  R  T  L  V  T  F  K  D  V  F  V  D  F  T  R  E  E  W  K  29
1     ACCATGGATGCTAAGTCACTAACTGCCTGGTCCCGGACACTGGTGACCTTCAAGGATGTATTTGTGGACTTCACCAGGGAGGAGTGGAAG
      L  L  D  T  A  Q  Q  I  V  Y  R  N  V  M  L  E  N  Y  K  N  L  V  S  L  G  Y  Q  L  T  K  59
91    CTGCTGGACACTGCTCAGCAGATCGTGTACAGAAATGTGATGCTGGAGAACTATAAGAACCTGGTTTCCTTGGGTTATCAGCTCACTAAG
      P  D  V  I  L  R  L  E  K  G  E  E  P  W  L  V  E  R  E  I  H  Q  E  T  H  P  D  S  E  T  89
181   CCAGATGTGATCCTCCGGTTGGAGAAGGGAGAAGAGCCCTGGCTGGTGGAGAGAGAAATTCACCAAGAGACCCATCCTGATTCAGAGACT
      S  R  T  S  G  S  Y  S  Q  A  P  S  Q  Y  S  Q  Q  S  S  S  Y  G  Q  Q  N  P  S  Y  D  S  119
271   TCTAGAACTAGTGGATCCTACAGCCAGGCTCCAAGTCAATATAGCCAACAGAGCAGCAGCTACGGGCAGCAGAACCCTTCTTATGACTCT
      V  R  R  G  A  W  G  N  N  M  N  S  G  L  N  K  S  P  P  L  G  G  A  Q  T  I  S  K  N  T  149
361   GTCAGAAGAGGAGCTTGGGGCAATAACATGAATTCTGGCCTCAACAAAAGTCCTCCCCTTGGAGGGGCACAAACGATCAGTAAGAATACA
      E  Q  R  P  Q  P  D  P  Y  Q  I  L  G  P  T  S  S  R  L  A  N  P  G  S  G  Q  I  Q  L  W  179
451   GAGCAACGGCCCCAGCCAGATCCGTATCAGATCCTGGGCCCGACCAGCAGTCGCCTAGCCAACCCTGGAAGCGGGCAGATCCAGCTGTGG
      Q  F  L  L  E  L  L  S  D  S  A  N  A  S  C  I  T  W  E  G  T  N  G  E  F  K  M  T  D  P  209
541   CAATTCCTCCTGGAGCTGCTCTCCGACAGCGCCAACGCCAGCTGTATCACCTGGGAGGGGACCAACGGGGAGTTCAAAATGACGGACCCC
      D  E  V  A  R  R  W  G  E  R  K  S  K  P  N  M  N  Y  D  K  L  S  R  A  L  R  Y  Y  Y  D  239
631   GATGAGGTGGCCAGGCGCTGGGGCGAGCGGAAAAGCAAGCCCAACATGAATTACGACAAGCTGAGCCGGGCCCTCCGTTATTACTATGAT
      K  N  I  M  T  K  V  H  G  K  R  Y  A  Y  K  F  D  F  H  G  I  A  Q  A  L  Q  P  H  P  T  269
721   AAAAACATTATGACCAAAGTGCACGGCAAAAGATATGCTTACAAATTTGACTTCCACGGCATTGCCCAGGCTCTGCAGCCACATCCGACC
      E  S  S  M  Y  K  Y  P  S  D  I  S  Y  M  P  S  Y  H  A  H  Q  Q  K  V  N  F  V  P  P  H  299
810   GAGTCGTCCATGTACAAGTACCCTTCTGACATCTCCTACATGCCTTCCTACCATGCCCACCAGCAGAAGGTGAACTTTGTCCCTCCCCAT
      P  S  S  M  P  V  T  S  S  S  F  F  G  A  A  S  Q  Y  W  T  S  P  T  G  G  I  Y  P  N  P  329
901   CCATCCTCCATGCCTGTCACTTCCTCCAGCTTCTTTGGAGCCGCATCACAATACTGGACCTCCCCCACGGGGGGAATCTACCCCAACCCC
      N  V  P  R  H  P  N  T  H  V  P  S  H  L  G  S  Y  Y                                    347
991   AACGTCCCCCGCCATCCTAACACCCACGTGCCTTCACACTTAGGCAGCTACTACTAGAAGCTT
```

Appendix 12

Human mutant KRAB/FLI-1 Fusion Gene

```
                                                                  M   1
                                                          CTTGGTTCGCCACCATG
     D  A  K  S  L  T  A  W  S  R  T  L  V  T  F  K  A  A  F  V  D  F  T  R  E  E  W  K  L  L  31
1    GATGCTAAGTCACTAACTGCCTGGTCCCGGACACTGGTGACCTTCAAGGCAGCGTTTGTGGACTTCACCAGGGAGGAGTGGAAGCTGCTG
     D  T  A  Q  Q  I  V  Y  R  N  V  M  L  E  N  Y  K  N  L  V  S  L  G  Y  Q  L  T  K  P  D  61
91   GACACTGCTCAGCAGATCGTGTACAGAAATGTGATGCTGGAGAACTATAAGAACCTGGTTTCCTTGGGTTATCAGCTTACTAAGCCAGAT
     V  I  L  R  L  E  K  G  E  E  P  W  L  V  E  R  E  I  H  Q  E  T  H  P  D  S  E  T  E  A  91
181  GTGATCCTCCGGTTGGAGAAGGGAGAAGAGCCCTGGCTGGTGGAGAGAGAAATTCACCAAGAGACCCATCCTGATTCAGAGACTGAAGCT
     S  R  T  S  G  S  Y  S  Q  A  P  S  Q  Y  S  Q  Q  S  S  S  Y  G  Q  Q  N  P  S  Y  D  S  121
271  TCTAGAACTAGTGGATCCTACAGCCAGGCTCCAAGTCAATATAGCCAACAGAGCAGCAGCTACGGGCAGCAGAACCCTTCTTATGACTCT
     V  R  R  G  A  W  G  N  N  M  N  S  G  L  N  K  S  P  P  L  G  G  A  Q  T  I  S  K  N  T  151
361  GTCAGAAGAGGAGCTTGGGGCAATAACATGAATTCTGGCCTCAACAAAAGTCCTCCCCTTGGAGGGGCACAAACGATCAGTAAGAATACA
     E  Q  R  P  Q  P  D  P  Y  Q  I  L  G  P  T  S  S  R  L  A  N  P  G  S  G  Q  I  Q  L  W  181
451  GAGCAACGGCCCCAGCCAGATCCGTATCAGATCCTGGGCCCGACCAGCAGTCGCCTAGCCAACCCTGGAAGCGGGCAGATCCAGCTGTGG
     Q  F  L  L  E  L  L  S  D  S  A  N  A  S  C  I  T  W  E  G  T  N  G  E  F  K  M  T  D  P  211
541  CAATTCCTCCTGGAGCTGCTCTCCGACAGCGCCAACGCCAGCTGTATCACCTGGGAGGGGACCAACGGGGAGTTCAAAATGACGGACCCC
     D  E  V  A  R  R  W  G  E  R  K  S  K  P  N  M  N  Y  D  K  L  S  R  A  L  R  Y  Y  Y  D  241
631  GATGAGGTGGCCAGGCGCTGGGGCGAGCGGAAAAGCAAGCCCAACATGAATTACGACAAGCTGAGCCGGGCCCTCCGTTATTACTATGAT
     K  N  I  M  T  K  V  H  G  K  R  Y  A  Y  K  F  D  F  H  G  I  A  Q  A  L  Q  P  H  P  T  271
721  AAAAACATTATGACCAAAGTGCACGGCAAAAGATATGCTTACAAATTTGACTTCCACGGCATTGCCCAGGCTCTGCAGCCACATCCGACC
     E  S  S  M  Y  K  Y  P  S  D  I  S  Y  M  P  S  Y  H  A  H  Q  Q  K  V  N  F  V  P  P  H  301
810  GAGTCGTCCATGTACAAGTACCCTTCTGACATCTCCTACATGCCTTCCTACCATGCCCACCAGCAGAAGGTGAACTTTGTCCCTCCCCAT
     P  S  S  M  P  V  T  S  S  S  F  F  G  A  A  S  Q  Y  W  T  S  P  T  G  G  I  Y  P  N  P  331
901  CCATCCTCCATGCCTGTCACTTCCTCCAGCTTCTTTGGAGCCGCATCACAATACTGGACCTCCCCCACGGGGGGAATCTACCCCAACCCC
     N  V  P  R  H  P  N  T  H  V  P  S  H  L  G  S  Y  Y                                    349
991  AACGTCCCCCGCCATCCTAACACCCACGTGCCTTCACACTTAGGCAGCTACTACTAGAAGCTT
```

Appendix 13

Mus musculus stromelysin-1 gene, promoter region

Accession number: AF077676

caccaagcacaacccttattctcacaccagcataaacaaatattccgcct
tttttgttcagggaagacaaaacacagattttgtttcatctaaggaataa
aaaattatagacctgtttttgagtggtctttaaagagaactcggaatgga
aatggatgccttattgtgatgtgatgttcggtctctggccaactgtctct
gtcaggcatttgcagtactggggatgtattccacaaaaacagtaaccctg
atctttgtacatcttcccaactgaaaatataaattgcacagaaagaaaat
gtgagaaagaagaacaaagagagagaaaatgcagatcctatatgtgcaca
gcatgtagttcaccataatgtgactctcatcgacagcatagtgtgctcat
taggaaacaaatggctgacaaacaagcaaattaaaagcacgcaatgtgtg
gtgttgggaaaactggagaaggaggctggaaaggggcaagggtggctaag
tttagcaaaaccttttataaaatgcttaagtactacagtcatgcattata
cactgagaatacaagtgagactctactggggaggggagggaaggagaggg
gaggggagagagagaggaaaagaggtgaggcaaatggagggaaggagaga
atagggggaggagtaaagaagagagaagaggagaagcaaattaaagtaaag
cataagcaaagaatcttgggtgatatttttcaacatcaaatcatctacta
caaaagaaaacttgtgacaaaaataaaagatatgagataaaccaggacat
tctagttcagtaaatcatatcaatatatgagtctttatagaaaaaggtat
tataggcccatgatcttttaatatatgtggtcactgatagtgtggactgt
agctatacatgcagacattttccttacctcttctcacagtattcagcttg
ggcttctggaagttctttgtacaacttggacttttttaccaagttagtcca
cttctatccaagtcacaaacattacagctctggaaggacagttaaatttt

ETS1 and ETS2 Binding Site

ccaaagtggaaaaaaatgccccagtttttctcttttgccaagGCAGGAAG
CATttcctggagattaatcaccatttgccttgcaaaattaagaaggtttg
aagaacttagtaaagaagattgtatcaccctactctgatttttaatttttt

Tel Binding site

GGAAgtggtcccatttggatggaagcaattatgagtcagttttcgggtga
ctctacaaacacaaccactctataaaagttgggcttaagaaggtggacct
agaaggaggcagcagagaacctactgaaggtggtacagagctgtgggaag
tcaATGaaaATGaaggg

Appendix 14

Genomic Sequence of murine *EWS* exons 6 to exon 8

```
ACCACAGGATGGTAACAAGCCTGCTGAGACTAGCCAACCTCAATCTAGCACAGGGGGTTATAACCAACCCAGCCTAGGATATGGACAGAG   90
TAACTACAGCTATCCCCAGGTACCTGGAGCTACCCAATGCAGCAGTCACCGCACCTCCATCTTATCCTCCTACCAGGTTAGTCTCATT  180
TTTTGTGGCCGCTGAATTAGTGATAATAAGACACATATTCTCTTTTCAGGTTGTTGACTTGCTAGCATTGGCTTCCTTTTTTTTGTTTTG  270
TTTTGTTTTGTTTTTCGAGACAGGGTTTCTCGGTATAGCCCTGGCTGTCCTGGAACTCAATTTGTAGACCAGGCTGGCCTCGAACTCAGA  360
AATCCGCCTGCCTCTGCCTCCCAAGTGCTGGGATTAAAGGCATGCGCCACCACGCCCAGCTTAGCGTTGGCTTCCTTGGATAAGATTTGT  450
TTTCTGACTTGCCCATAGTTGACCACATTCCTAGTGAATAGTCACACCTCAGCCTTGGATTCCCATAGCAGTGCTTGGGATCTAGCTAAT  540
AACTGTTCTCTAAATACCGACCGCAAAACTTCAGTTTTGGTCTTATAACTAACTTAAAAGTATGAGAAACAGAACTGTTTGGAGCCATTG  630
GCACAGAACCATAGCAATTACTCCTCAAACACAGGCACCATGGGCAAAGTCAAGGGCAAGACCTCCCTACTACTGTTTGTAGTGCTGGGG  720
AGGGAATCTGGGCTCTTGTTCATTGCTGGGAAAGGACTTTGCCTATGAAGTACTCCAGCTCAAGTTTCCTTGTCCTTCATTTTCTGTCTT  810
AACCATTGTAGCTTCAATGTTCAATTTTAGTTTAGAAAGCTGCAAGTTTTCTGAGCTGTGTCTCATTTGAAGTAAGTTTCAAATGAATTG  900
ACAAAATTGTTTCAAAAGGTTAGTTTAATTGGCTATAATATAAAATAGTTTCTGTTTGCATCTGTTTTAGGGTTTCTAGTCCTGCACAAA  990
ACATCATAACTAAGAAGCAAGTAGGGGAGGAAAGGGTTTATTCGGCTTACACTTACTTTGCTGTTCATCACCAAGGAAGTCAGAACTGGA 1080
ACTCACACAGCGCAGGAAACTGGAGGCCCGAGCTGATGCAGAGGCCATGGAGGGGTGCTGCTTACTGGATTGCTTCCCCTGGTTTGCTCA 1170
GCTTGCTTTCTTACAGAACCCACAATAGGCTGGGCCCTCCCCTCTTGATCACTAATTGAGAAAATGTCTTACAGCTGGGTCTCATGGAGG 1260
CATTTCCTCAATGGAGGCTTCTTTTGTTTTTTTTTTTTTTTTTTTTGAAATATTTATTTATTATATGTAAGTACACTGTAGTGTGTCTTC 1350
AGACACTCCAGAGGAGAGCATCAGATCTCATTACGGATGGTTGTGAGTCACCATGTGGTTGCTGGGATTTCAACTCAGGAACTTTGGAAG 1440
AGCAGTTAGCCCTCTTAACCACTGAGCCAGCTGACCAGCCCCTTCTTTGTTTTGTTAAGATTATTTTATTTATATGAGTGCACTCTGTC 1530
TTCAGACATTCCAGAAGAGAGCATACAGGTTGTGGGCCATCATGTGGTTGCTAGGAATTAAATTCAGGACCTCTGGAAGAGGAGCCATCT 1620
CTCCAACCCCATAGACTGAATCTTGACAGGTGTGGAGAGGTGTGACAAATACTTAGTTTAGTAGAACATATTTATGGTTCATTGAGAGTC 1710
CCATTGTGTCCCTACTCCAGCCAGGGTTATTCTGTGTGGTCCTGACTGTCCTAAAACTCAATCTAGATTATTTAGGACAGGCTGGCCCTGA 1800
ACCCAGGGATCTGTCTGCCTCTTGGGAGACAAGAGTCCTGGGATTAAAGCCTTGCCTTGCCACTACACCCAGCTAAGAGTCTTTAAGGTT 1890
TATTTTTAGTAGTATGTGGAGGTACATAGATTACCTGCTGGGTAGTTTTACAGAGACCTAACATCTTTTATACAGTATCCTAAGGTCTCT 1980
TACCAGTCTCGGTCTTTAATTCCTAGATTTCCCAAGGATCAAATATGTTTGGTTTGAAAGGTTGAAACTATAGCAAAGCGATTTTGAGTC 2070
TGAAAGAAAATGTCATTTTTGGAGTGGCATACTTTAATATGCTCATGTTTACACTCTATTACCAAGCAGGTATTCTTCAGAAAGTCATAG 2160
TCATAACTCAACCTATTAGACGGGCTGAAATAGAACACTTGCTAGTGTGTGAGGCTCTAAGTTGGAGCCTCAGAACTACAAAAAGAAAAG 2250
AAGGTGTTCTGGGAACTAAATACCTCACTTGACTCTTCTTCCTCCTCACTTTCCGTGTCTTTTCATTAGTCATCTCTTAAGTGCACATGT 2340
ACATAGTCTGCCCTTCTGAACTGTATTTGTCTTAAATGTATTTGAAAAACGTTTTAAGTAGGAGCTCTGAACCACAACTCAATGTTCACA 2430
TGGGTTCTTTTCATTTTCTCTTTTACATTTAAAGCTTTATTATTGGAAATAAATCCAAAAGTGTTGCCAGACAGTGTTGCACACCTGTAA 2520
TCCAGCACACGGGAAACTGAGGCAGAAGAGTTGTGTGCAGGTCGACAGTCCGCCTGGGCTACATGTAGTAGACCCTCTCAATGAAAGTA 2610
AAAAGAAAGTAATTGTTAGGATGCCTGTGAATGTCTCATATTCTTGGCAGAAATAACATGATTTTTCTCCTTCCTGAAGATTCTGTTTTT 2700
TTCTTTCAGCTACTCCTCTTCACAGCCGACTAGTTACGATCAGGCAGTTACTCTCAGCAGAACACCTATGGGCAGCCGAGCAGCTATGG 2790
ACAACAGAGTAGCTATGGTCAACAATGCAGCTATGGGCAGCAGCCTCCTACTAGTTACCCCCCTCAGACTGGATCCTACAGCCAGGCTCC 2880
AAGTCAATATAGCCAACAGAGCAGCAGCTACGGGCAGCAGAGTGAGTTGCTAACAGAGAAAACTAAGTATCAGTGATTTTGCTTGGAGTT 2970
TGTGAGACTGGGGAATGCTTGTTACCACTGTTTGCTTCTGGAGTCTAGAGTGTGAGTGTGCACTATAACATCTGATCCTGCATGATAGTC 3060
TGAATGCCCAAGTAGGGGCTCTGTCAGCAGGGTGGGCGCTCTGCAGTGAGTAGAGCTGGCATTCTTTAAACCTGCCTTAATTAAACATTA 3150
AGAGAAAGTGATAGCTATACAGGGCTACTGTGGATTTTGATAACCAATTAAAAACTGTGTTTTGATTTTGGGTAACTGATTGAGCAAAGG 3240
GAGTCCTGCTCACAGAGCACTAGCGAGCACACCCCCAACCCCCAGTGCTTATCCTTACATTTGGAAAAGTGACTAAAAACCCAAAGTAT 3330
CTCAGTCACTTTTTAATTTCTGCCTCAATTGTTGCACTTTTAAATTTGAATTTCATCAGCCAGGCATGATGGCGCAGCATTTGCAGTGAG 3420
GTTCCGACTCTTAAGGCTCTTAAGGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTTGTTTTATTTGTTGCTGGGAT 3510
GACTAGTTACAATTCCGCCCTTTGTACGCCAGAGCCCTAGGTAACTTGGCACTCACTGCTTTTGGTGTTGAGAGTGCAAAGAAGCTGCTT 3600
TTGGAGACATCTGAAGCACAGAAACTTACAAATATATTTTCCCAGAAGAAAAATCCTTTGGAAGTAGTTTTATAGCCTGGCCATACAAGG 3690
CCTTGATAAGCCAACAGTTCTTACAGAGAGAGAGCCTGTGATAGAAATACTTTGAGCATAGTGCTTATGTTGAGAGCTCCATGAGTACAG 3780
GAGAAAGGGTGTCAGGCATTGGTGTGAATGCTGGTCCGTGGCTTACAGATGTGACTCTTTCCTCAGGTTCATTCCGCAGGACCACCCCA 3870
GTAGCATGGGTGTTTATGGGCAGGACTCTGGAGGATTTTCCGGACCAGGAGAGGACCGGAGCTTGAGTGGCCGTGATAACCCGGGCAGGG 3960
GAAGAGGGGGATTTGATCGTGGAGGCATGAGCAGAGGTGGGCGGGGAGGAGGACGCGGTGGACTGGG                          4027
```

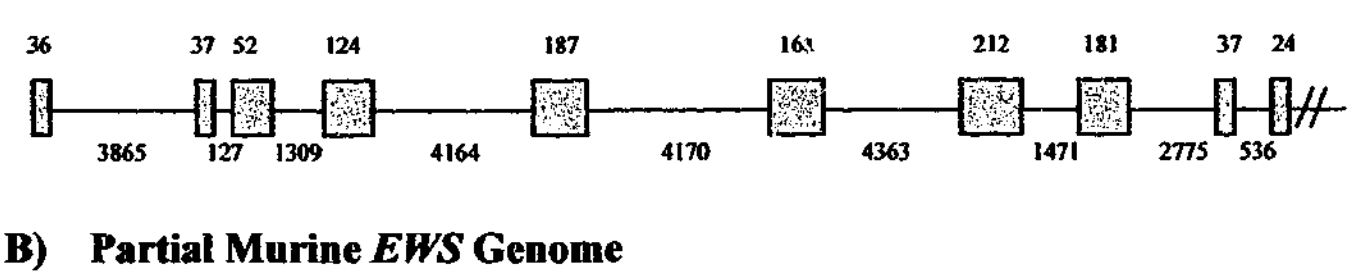
Exon 6

Exon 7

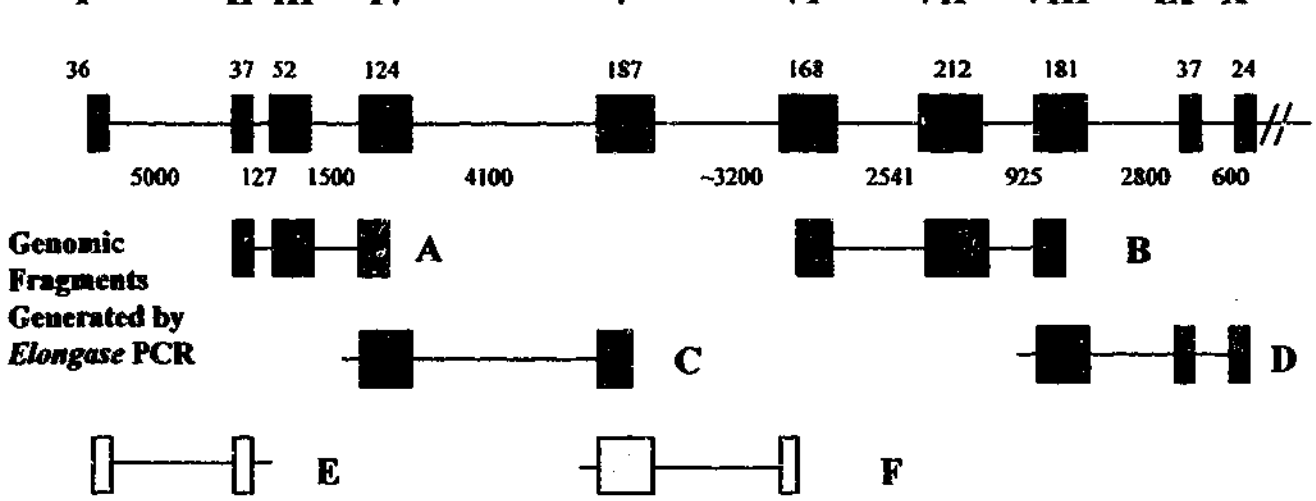
Exon 8

ERRATUM

A) Partial Human *EWS* Genome



B) Partial Murine *EWS* Genome



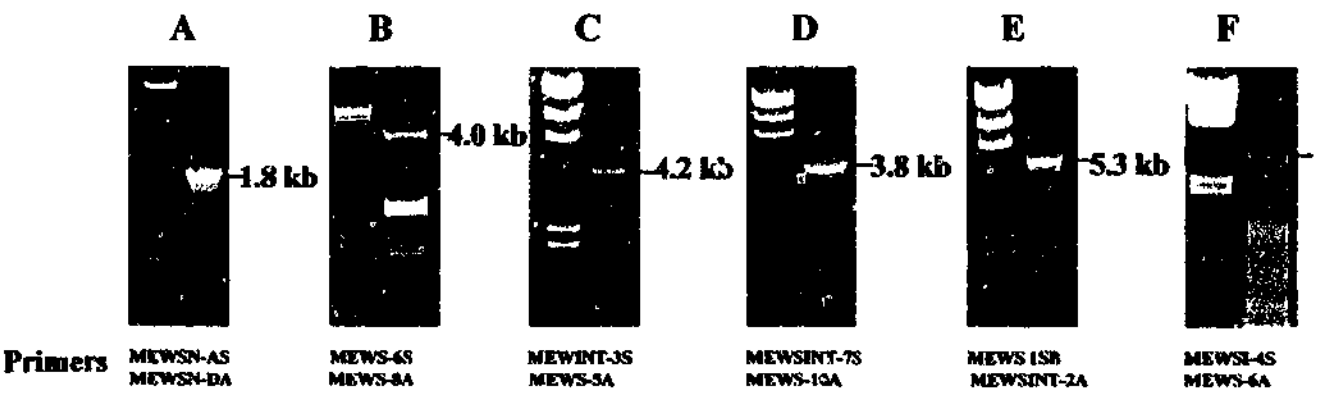
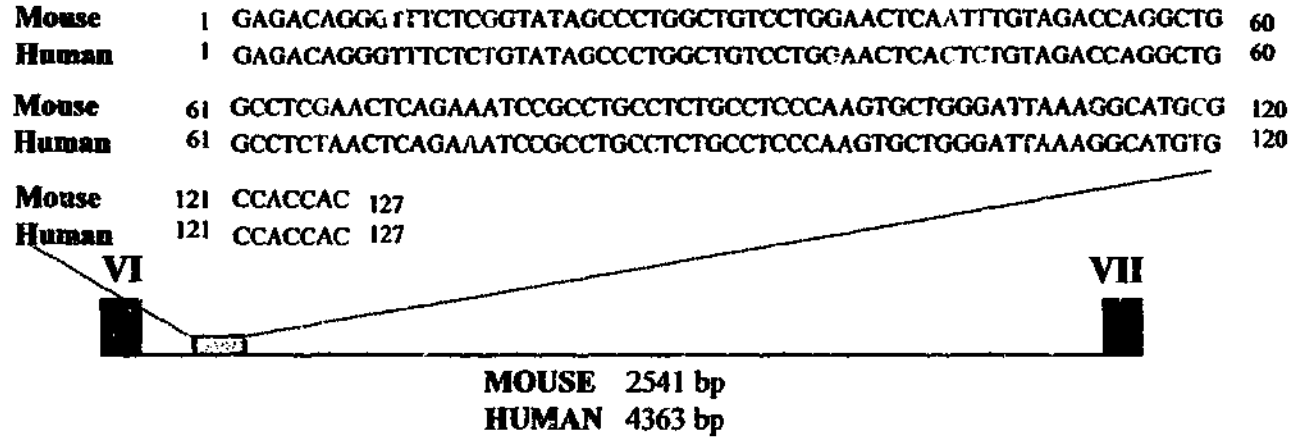


Fig.6.5 Schematic diagrams show the partial genomic structure (exons 1-10) of the human and murine *EWS* genes. (A) Partial human *EWS* genome. (B) Partial murine *EWS* genome and the Elongase PCR amplified fragments used for knockout and knock-in constructs. The exons are shown to scale as solid boxes. The lines represent introns and the numbers represent base pair (bp). The photos show the amplified genomic fragments on agarose gel.

ERRATUM

A



B

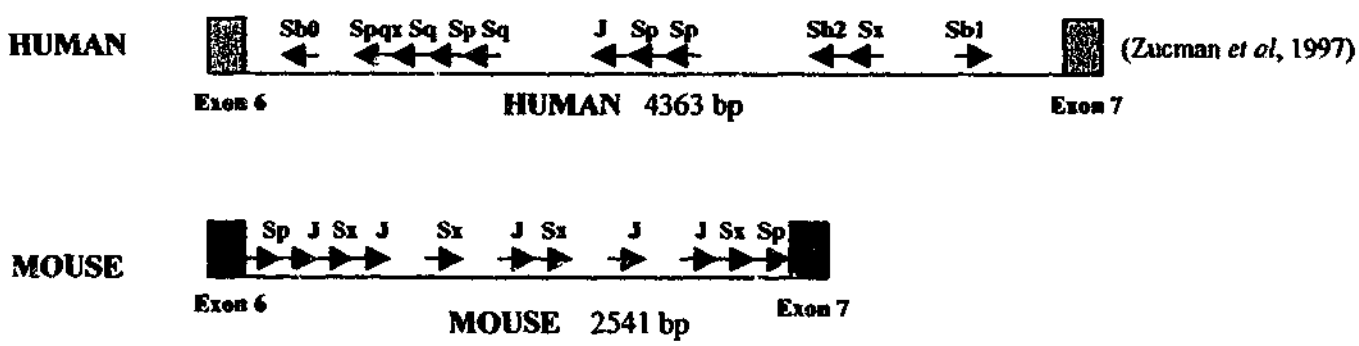


Fig.6.6 Comparison of intronic similarities between human and mouse *EWS* introns 6 and 7. (A) The comparison of sequence of some high identity regions in mouse and human *EWS* introns 6 and 7. (B) Distribution of the *Alu-like* B1 repeats and *Alu* repeats in mouse and human *EWS* intron 6 respectively (human data was extracted from Zucman *et al*, 1997).

ERRATUM

PAGE	SECTION	PARA	LINE	READS	SHOULD READ
80	4.2-1	1	7	transcription initiation complex	translation initiation complex
80	4.2-1	1	8	transcription	translation
80	4.2-1	1	8,10,12,13	KOZAK	Kozak
125	7.5-4	entire page			as shown below

Modified Figures 6.5 and 6.6 are attached.

4. Isolation and Characterization of Full-length of ER99

The identification, isolation and characterization of the full-length *ER99*, will be continued using 5' RACE PCR on ZR-75-1 cell line. From our preliminary data we would expect this gene to be oncogenic, thus the full-length *ER99* cDNA will be stably over-expressed in NIH3T3 cells to study its tumourigenicity. The expression of this gene within transformed cells will be further studied using more variety of human cancers and using human cancer tissue arrays. Production and analysis of a mouse lacking *ER99* could also be performed to elucidate the normal function of this gene.

Since this gene appears to be expressed in many cancer cell lines including ES/PNET, the promoter of *ER99* will be amplified according the full-length cDNA of *ER99* and genomic sequence of Human DNA from overlapping chromosome 19 cosmids R31396, F25451 (Accession number: AC002115). Putative *EWS/FLI-1* binding sites in the promoter region will be examined for direct regulation by *EWS/FLI-1* and/or *KF*. Furthermore, this promoter could be coupled to a "suicide gene" and transfected into normal and cancer cell lines to demonstrate inhibition of cancer cell growth. These data will indicate whether there is potential for use of this promoter in novel gene therapy.

ADDENDUM

Fig. 1.1 The bright regions (A-D) reveal multi-layering of the reactive bone around the tumor. Under light microscope, Ewing's sarcoma (E-F) consists of densely packed uniform small cells in sheets. The cells have scant cytoplasm without distinct borders. The cells have a single oval or round nucleus without prominent nucleoli.

Page 18 Section 1.4-2 Paragraph 2 Line 7. Pointed domain occurs in approximately one-third of ETS proteins. It is proposed to mediated protein-protein interactions and to be regulated by *ras*-dependent signaling because of the presence of an adjacent mitogen-activated protein (MAP) kinase phosphorylation site (Graves et al, 1998).

Graves, B. J. & Petersen, J. M. Vande Woude, G. & Klein, G., eds. (1998) *Advances in Cancer Research* (Academic, San Diego).

Table 1.6 The regions of different ETS transcription factors represent their expression pattern in different organs during the development of mouse.

Fig 3.7 and Fig. 4.11. The semi-quantitative RT-PCR for each samples were as follows: the first-strand cDNA of every samples were synthesized by using Reverse Transcription System (Promega) on 1 μg total RNA. One in 100 dilution first-strand cDNA sample was subjected to 25 cycles *Taq* polymerase amplification using specific primers.