Studying the Biological Function of the Fps/Fes Proto-Oncogene 
by Generating and Analyzing Transgenic Mice Expressing a 
Targeted Inactivating Mutation in the gene 

by 

Jennifer Lyn McVeigh 

A thesis submitted to the Department of Pathology 
in conformity with the requirements for 
the degree of Master of Science 

Queen’s University 
Kingston, Ontario, Canada 
September 1997 

copyright © Jennifer Lyn McVeigh, 1997
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-22357-4
SUCCESS

The talent of success is nothing more than doing what you can do well
and doing well whatever you do.

Longfellow
ABSTRACT

This thesis describes the investigation of the biological function of the fps/fes proto-oncogene, which encodes for a 92 Kd cytoplasmic tyrosine kinase, by generating transgenic mice which have a “loss of function” mutation in the endogenous locus. A point mutation was engineered in the cloned murine fps/fes gene; wild type and mutant fps/fes constructs were expressed in transfected cells, and the resulting lysine to arginine (K to R) amino acid substitution was shown to essentially abolish the catalytic activity of Fps/Fes. A targeting vector was also generated to introduce this mutation into mouse embryonic stem (ES) cells and several ES clones were then isolated which carry the mutation. Chimeric mice were produced and germline transmission has yielded viable homozygous mutant mice.

This thesis illustrates the initial analysis of the biological phenotype of the transgenic mice with reference to the tissue-specific expression pattern of the fps/fes gene. In order to distinguish between Fps/Fes and the closely related Fer kinase, highly specific polyclonal antisera were generated and subsequently used to analyze expression and activity of Fps/Fes and Fer in normal and transgenic mice. Initial analysis of the transgenic mice suggested that Fps/Fes activity is not essential for angiogenesis/vasculogenesis, as mice homozygous for the mutation in Fps/Fes were viable. There was no overt phenotype; histological analysis suggested that the mutation didn’t cause any gross morphological changes in any of the tissues examined. Upon closer examination, female fertility was abnormal, indicating potential uterine or placental defects. To investigate the effects of the mutation on hematopoiesis, initial methylcellulose assays suggested that Fps/Fes activity is not required for myeloid differentiation, although specific cytokine signaling pathways may be affected. Finally, the catalytically inactivated Fps/Fes protein was shown to be expressed (at appropriate levels) in the transgenic mice, and there may be an increase in Fer activity in the homozygous K to R mice. As Fer may be compensating for the loss of Fps/Fes activity, a ‘double knockout’ with Fer may reveal phenotypes that were masked in the single Fps/Fes K to R ‘knockout’.
ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Peter Greer for all his guidance and assistance throughout my Master's degree. I would also like to thank members of the Pathology department: Dr. David Lillicrap, Dr. Lois Mulligan, Dr. Sandy Boag and Dr. Sam Ludwin for their suggestions and advice at different times during my research.

I would like to thank Ralph Zirngibl for his knowledgeable input into my project and his technical help. Thanks to Marion Arnold, Rob Leggett, and Karen Williams for all their technical assistance. Thanks to past and present lab members: Lesley-Ann Cole, Denis Kim, Diane Bovenkamp, Tony Shearing, Karen Ambus, and Jody Haigh, for their cooperation and entertainment throughout the years.

Finally, I would especially like to thank Jay and my parents for all their support and encouragement.

SUPPORT

This work was supported by grants from the National Cancer Institute of Canada, the Medical Research Council of Canada and the Department of Pathology at Queen's University.
TABLE OF CONTENTS

ABSTRACT ....................................................................................................................... i

ACKNOWLEDGEMENTS ................................................................................................. ii

TABLE OF CONTENTS ................................................................................................... iii

LIST OF TABLES ............................................................................................................... v

LIST OF FIGURES ........................................................................................................... vi

LIST OF ABBREVIATIONS ............................................................................................... vii

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW .......................................... 1

Nonreceptor or cytoplasmic protein tyrosine kinases ......................................................... 1

Fps/Fes ............................................................................................................................... 2

Expression ......................................................................................................................... 4

Transcriptional Regulation ............................................................................................... 4

Functional Domains ......................................................................................................... 5

Role in vascular endothelial signaling pathways ............................................................... 6

Role in hematopoietic signaling pathways ....................................................................... 7

Factors mediating Fps/Fes signaling ................................................................................. 8

Hematopoiesis .................................................................................................................... 11

Vasculogenesis and Angiogenesis ..................................................................................... 13

Fer .................................................................................................................................. 16

Knockouts ........................................................................................................................ 16

Experimental Rationale .................................................................................................... 17

CHAPTER 2: MATERIALS AND METHODS

Generation of rabbit polyclonal antisera ............................................................................. 20

Affinity purification of polyclonal antisera ........................................................................ 24

Cos-1 cell culture and transfection .................................................................................... 25

Harvesting Cos-1 cells ........................................................................................................ 26

Western blotting ................................................................................................................ 27

Preparation of mouse tissue lysates .................................................................................... 28

Immune complex kinase assays ........................................................................................ 28

Generation of expression constructs and PCR mutagenesis .............................................. 29

Generation of targeting vector .......................................................................................... 31

ES cell culture and electroporation .................................................................................... 32

Screening ES cell clones ..................................................................................................... 34

Genomic Southern blotting ................................................................................................ 36

Generation of chimeric mice ............................................................................................... 38

Genotyping of mice ............................................................................................................ 40

Histological examination of mouse tissues ....................................................................... 41
**CHAPTER 3: RESULTS**

Generation and Characterization of polyclonal antisera ........................................ 4 4
   Cos-1 cell transfection analysis ........................................ 4 4
   Mouse tissue Western blots ........................................ 4 9
Generation of the "loss of function" mutation .................................................... 5 2
In vitro characterization of the mutation ....................................................... 5 2
In vivo characterization of the mutation ....................................................... 5 5
   Targeting strategy ................................................... 5 5
   PCR screening of ES cell clones ........................................ 5 7
   BgIII genomic southern blot analysis of ES cell clones .................... 5 9
Generation of chimeric mice ................................................................. 6 1
F1 and F2 generations of mice ............................................................... 6 1
Phenotypic analysis of transgenic mice ..................................................... 6 6
   Histological examination .................................................. 6 6
   Fertility of homozygous mutant mice ....................................... 6 8
   Hematopoiesis/Methylcellulose assays ........................................ 7 0
   Western blots and Immune complex kinase of bone marrow
      from transgenic mice .................................................. 7 2

**CHAPTER 4: DISCUSSION**

Implications of generating and characterizing the anti-Fps
   and anti-Fer antisera ................................................ 7 5
Generation of transgenic mice ............................................................... 7 5
Phenotype and Future perspectives .......................................................... 7 8
   Role of Fps/Fes in blood vessel formation .................................... 7 8
   Role of Fps/Fes in hematopoiesis ............................................ 7 9
   Role of Fps/Fes in secretion ................................................ 8 0
   Role of Fps/Fes in pregnancy ................................................ 8 0
   Cytokine signaling pathways ..................................................... 8 2
   Functional redundancy with Fer ................................................ 8 2

**REFERENCES** ................................................................. 8 4

**CURRICULUM VITAE** ............................................................ 9 3
LIST OF TABLES

Table 1: Summary of the reactivity of the different antisera generated. . . 48

Table 2: Summary of expression of Fps/Fes and Fer proteins in a number of mouse tissues. ................................. 51

Table 3: Chimeras generated from ES cell lines with targeted K to R mutation in fps/fes. ................................. 62
LIST OF FIGURES

Figure 1: Nonreceptor or cytoplasmic protein tyrosine kinases. ............... 3
Figure 2: Factors implicated in mediating Fps/Fes signaling. ................. 10
Figure 3: Hematopoiesis. .................................................. 12
Figure 4: Blood vessel formation: vasculogenesis and angiogenesis. ....... 14
Figure 5: Illustration of concept of functional redundancy. ................. 18
Figure 6: GST-fusion proteins used to generate polyclonal sera. ............ 21
Figure 7: Generation of expression and targeting constructs. ............... 30
Figure 8: Illustration of the procedure used to generate transgenic mice. .. 39
Figure 9: Specificity of the polyclonal antisera generated against the various regions of the Fps/Fes and Fer proteins. .................. 45
Figure 10: Immunoprecipitations of Fps/Fes or Fer proteins from fps/fes or fer transfected Cos-1 cells. ......................... 47
Figure 11: Fps/Fes and Fer protein expression in various mouse tissues. .... 50
Figure 12: In vitro characterization of the lysine to arginine mutation. .... 54
Figure 13: Targeting Strategy ............................................. 56
Figure 14: Southern Blot Analysis of the PCR screen on ES cell clones. .... 58
Figure 15: BglII genomic Southern Blot analysis. .......................... 60
Figure 16: Transgenic mice which have been generated (Chimeras and the F1 generation). ................................. 63
Figure 17: Transgenic mice which have been generated (the F2 generation and adult females of each genotype). ............. 64
Figure 18: PCR genotyping of transgenic mice. .......................... 65
Figure 19: Histological examination of the lung. .......................... 67
Figure 20: Examination of female fertility in +/- K to R mice. .............. 69
Figure 21: Evidence that hematopoiesis is 'normal' in +/- K to R mice. ...... 71
Figure 22: Evidence that the transgenic mice express the inactivated kinase. .. 73
LIST OF ABBREVIATIONS

µg- microgram
µl- microliter
µm- microns
µM- micromolar
%- percentage
A- alanine
AP- affinity purified
approx.- approximately
ATP- adenosine triphosphate
BCR- breakpoint cluster region
BFU-E- burst-forming unit-erythroid
BSA- bovine serum albumin
c-src- cellular form of the gene found in Schmidt-Ruppin strain of Rous sarcoma virus
Cl66 cell line- an endothelial cell line, derived from 'MF' transgenic mice
Cas- catenin-associated substrate
cDNA- complementary deoxyribonucleic acid
CFU-E- colony-forming unit-erythroid
CFU-GM- colony-forming unit-granulocyte-monocyte/macrophage
Cos-1- monkey kidney cell line
CSF-1- colony stimulating factor-1
D- aspartic acid
dATP- deoxyadenosine triphosphate
dCTP- deoxycytosine triphosphate
dGTP- deoxyguanine triphosphate
DMEM- Dulbecco’s modified Eagle’s media
dNTPs- deoxynucleotide triphosphates
DTT- dithiothreitol
dTTP- deoxythymine triphosphate
E- glutamic acid
Epo- erythropoietin
ES cell- embryonic stem cell
EtBr- ethidium bromide stain
FBS- fetal bovine serum
Fer- Fps/Fes-related kinase
ferT- testis specific fer transcript
FGF- fibroblast growth factor
Flk-1- fetal liver kinase-1
Flt-4- Fms-like tyrosine kinase-4
Flt-1- Fms-like tyrosine kinase-1
Fms- CSF-1 receptor
Fps/Fes- fujinami poultry sarcoma/feline sarcoma
fyn- src family kinase
G- glycine
G418- geneticin, a neomycin analog
GANC- gancyclovir
GAP- ras GTPase-Activating Protein
GM-CSF- granulocyte-macrophage colony stimulating factor
GRB-2/SOS- guanine nucleotide exchange complex
GRB-2- growth factor receptor-bound protein
GST- glutathione-S-transferase
H or His- histidine
I- isoleucine
IL-3- interleukin-3
IL-6- interleukin-6
IPTG- isopropyl β-D-thiogalactopyranoside
JAK- Janus Kinase or Just Another Kinase
K to R- lysine to arginine amino acid substitution
K- lysine
Kd- kilo-dalton
KLB- kinase lysis buffer
KRB- kinase reaction buffer
L- leucine
M-CSF- macrophage colony stimulating factor
M- molar
MF- myristylated Fps/Fes
mg- milligram
min- minute
ml- milliliter
mM- millimolar
mRNA- messenger ribonucleic acid
N- asparagine
N-terminal domain- amino-terminal domain of the protein
N-Fps- amino-terminal region of the Fps/Fes protein
neo- neomycin resistance gene cassette
O/N- overnight
oligo- oligonucleotide
P92- protein of 92000-daltons, Fps/Fes
P94- protein of 94000-daltons, Fer
PAGE- polyacrylamide gel electrophoresis
PBS- phosphate buffered saline
PCR- polymerase chain reaction
PDGF- platelet-derived growth factor
PMSF- phenylmethyl sulfonylfluoride, a protease inhibitor
prehyb- prehybridization solution
PU.1- DNA binding protein that binds to the PU box (purine-rich sequence)
pY- phosphorylated tyrosine residue
Q- glutamine
R- arginine
ras- a guanine nucleotide-binding protein
RNase- ribonuclease
rpm- revolutions per minute
rt- room temperature
SCF- stem cell factor
SCL/tal-1- a basic-helix-loop-helix transcription factor
SDS-PAGE- sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS- sodium dodecyl sulphate
SH2- src homology 2
Sp1- sephacryl phosphocellulose factor 1, a promoter-specific transcription factor
SSC- standard saline citrate
Stat- Signal Transducers and Activators of Transcription
TBS- tris buffered saline
TBS-T- tris buffered saline with 0.1% Tween-20
Tie-1 or Tie-2-
tk- herpes simplex thymidine kinase gene
V- valine
VEGF- vascular endothelial growth factor
X- any amino acid
Y- tyrosine
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

This thesis illustrates investigations into the biological function of the *fps/fes* proto-oncogene. *Fps/Fes* belongs to the nonreceptor or cytoplasmic protein tyrosine kinase family of which the Fer tyrosine kinase is the most closely related member. Initially, cytoplasmic tyrosine kinases will be introduced in general. *Fps/Fes* will then be discussed with reference to its expression pattern, transcriptional regulation, functional domains, and the role *Fps/Fes* plays in vascular endothelial and hematopoietic signaling pathways. Since *Fps/Fes* is thought to play a role in hematopoiesis, the process by which all mature blood cells arise, and blood vessel formation (vasculogenesis and angiogenesis), these processes will then be reviewed. The Fer tyrosine kinase will be examined. Finally, 'knockouts' and how this experimental technique may aid in the determination of the biological function of a gene will be introduced. The experimental rationale will be stated. This thesis describes investigations into the biological function of the *fps/fes* proto-oncogene that were performed by generating and analyzing transgenic mice expressing a targeted inactivating mutation in the gene.

Nonreceptor or cytoplasmic protein tyrosine kinases

In contrast to receptor protein tyrosine kinases, nonreceptor or cytoplasmic tyrosine kinases lack transmembrane and extracellular domains. Members of the cytoplasmic tyrosine kinase group were initially discovered as proteins encoded by dominant acting oncogenes of transforming retroviruses (reviewed by (1)). Currently, there are eight different families with several common major domains: the catalytic or
SH1 (src homology 1) domain, which is involved in the phosphorylation of tyrosine residues on specific substrates in the cell (2), the SH2 domain (src homology 2), which allows interaction with selected phosphotyrosine containing proteins (3), and the SH3 domain (src homology 3), which allows interaction with proline-rich sequences in selected proteins (4) (see figure 1). The physiological roles of these enzymes, which are involved in a variety of cellular signal transduction pathways, are just beginning to be unraveled with the use of gene 'knockout' techniques in mice (reviewed in (5)).

**Fps/Fes**

The *fps/fes* proto-oncogene encodes a cytoplasmic protein tyrosine kinase of 92 Kd (6). *Fps/Fes* was originally identified as a retroviral oncogene capable of causing sarcomas in chickens and cats (7,8) thus it's name "fujinami poultry sarcoma virus/feline sarcoma virus". The cellular form of the *fps/fes* gene has been found on human chromosome 15 and mouse chromosome 7; these genes have been cloned, sequenced and shown to be highly homologous to the feline and chicken counterparts (9,10 and Zirngibl & Greer, unpublished). Although *fps/fes* expression has been seen in a number of human malignancies, such as carcinoma of the lung, breast and kidney, as well as acute and chronic myelogenous leukemias (11), there is no evidence of mutations or chromosomal translocations involving *fps/fes*, which correlate with Fps/Fes activation, in any human cancer.
Figure 1: Nonreceptor or cytoplasmic protein tyrosine kinases.

The eight different families of nonreceptor or cytoplasmic protein tyrosine kinases are illustrated. The common major domains: the catalytic or SH1 (src homology 1) domain, the SH2 and the SH3 domains, as well as the sizes of the proteins are shown. This figure was adapted from a review by J. B. Bolen (5).
Abl, Arg  

Jak1, Jak2, Tyk2  

Fak  

Fps/Fes, Fer  

Syk, Zap  

Itk (Tsk), Bpk (Atl), Tec  

Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Frg, Yrk  

CsK
Expression

Fps/Fes is thought to be involved in the regulation of cellular growth and differentiation in a variety of cell types. Early studies suggested that Fps/Fes was exclusively expressed in cells of hematopoietic origin, such as macrophages and granulocytes (6,12), but more recently fps/fes expression has been seen in rapidly proliferating human and mouse embryonic tissues originating from all three germ layers: ectoderm, mesoderm and endoderm (13,14). RNA in situ and immunohistochemistry analyses have shown fps/fes expression in cells of mesodermal origin, such as chondrocytes and vascular endothelial cells (15), in cells of ectodermal origin, such as neuronal cells, and in cells of endodermal origin, such as bronchiole epithelium (14). RNase protection studies have shown tissue specific expression of fps/fes in bone marrow, spleen, lung, and lymph nodes, with low expression in peripheral blood and the ovary (16). RNA in situ and immunohistochemistry analyses have also shown fps/fes expression in angioblasts in the yolk sac, in glandular epithelial cells of the uterus, in the sinusoidal endothelium of the central vein in the liver, in the highly vascularized marginal zones of the spleen, and in many areas of the brain including the epithelium of the choroid plexus (14). Fps/Fes expression was also shown in endothelial, neuronal, and epithelial cell lines (14); consequently, recent studies suggest fps/fes is more widely expressed than originally thought.

Transcriptional Regulation

Very few studies have been conducted analyzing the promoter of fps/fes. DNase I footprinting analysis revealed four distinct cis-acting elements in the 5' region of fps/fes that bind myeloid nuclear proteins and a weakly active region in the 3' region
Transcription factors Sp1 and PU.1/Spi1 were shown to bind the fps/fes promoter region by electrophoretic mobility shift assays (17,18). Mutating the PU.1/Spi1 binding site or the most 3' cis-acting element in the 5' region of fps/fes resulted in substantially reduced luciferase activity in transient transfection experiments (17). PU.1/Spi1 has also been shown to be required in the development of a variety of hematopoietic cell types; 'knockout' mice die one to three days before their expected date of birth with an absence of monocytes, granulocytes, and T and B lymphocytes (19). Pu.1/Spi1 is thought to be a hematopoietic specific transcription factor; consequently transcriptional regulation of fps/fes in all of the cell types in which it is found to be expressed is not clearly understood.

**Functional Domains**

The three major domains of the Fps/Fes protein include the N-terminal region (N-Fps), the SH2 domain, and the catalytic domain. N-Fps may have a variety of functions. The amino-terminal region may be involved in the cellular localization of Fps/Fes (20) and in protein-protein interactions via specific recognition sequences, such as those recently found for the cellular BCR protein (21). A coiled-coil domain has recently been found in the amino-terminal region of Fps/Fes, consequently N-Fps appears to be involved in dimerization or oligomerization of the protein (22). This oligomerization may be essential for autophosphorylation, which has been shown to occur as an intermolecular event (23), and activation of the protein. Fps/Fes activation by oligomerization and transphosphorylation is analogous to receptor tyrosine kinases, but until now this mechanism of Fps/Fes activation was not known.

The SH2 domain allows protein-protein interaction with specific tyrosine
phosphorylated substrates (3). The kinase domain is involved in the phosphorylation of tyrosine residues on Fps/Fes itself or other substrates in the cell; some of the peptide motifs important for catalysis include the GXGXXG motif which is involved in binding ATP and the HRDLAARN motif which is the catalytic loop (reviewed by (2)). In addition, the major tyrosine phosphorylation site of Fps/Fes, Y713, and an intact SH2 domain appear to be required for full catalytic activity of the protein (24,25). Cytoplasmic tyrosine kinases appear to preferentially phosphorylate peptides recognized by their own SH2 domains or closely related SH2 domains (26). For example, the optimal substrate peptide sequence for Fps/Fes has been shown to be E-E-E-I-Y-E-E-I-E (26) and the SH2 domain of Fps/Fes optimally recognizes peptides of consensus pY-E-X-V/I (27). It is suggested that cytoplasmic tyrosine kinases such as Fps/Fes may phosphorylate a substrate and then associate with that substrate through its' SH2 domain (26). This association may protect the substrate from dephosphorylation or allow for repeated phosphorylation; pY-SH2 binding may also alter the subcellular localization of the protein, and induce conformational changes that alter the catalytic activity of the interacting proteins.

**Role in vascular endothelial signaling pathways**

Accumulating evidence suggests that Fps/Fes plays an important role in vascular endothelial and hematopoietic signaling pathways. Although the precise biological function of Fps/Fes in these cells is unknown, the viral form of Fps/Fes has been shown to transform fibroblasts in culture (28), suggesting Fps/Fes plays a role in regulating cellular growth and differentiation. Transgenic mice expressing the viral form of *fps/fes* under the control of the beta-globin promoter develop tumors of lymphoid and
mesenchymal origin (29) and have cardiac and neurological abnormalities (30). In addition, an activated \textit{fps/fes} gene expressed in transgenic mice appears to increase the mitogenic signaling in vascular endothelial cells as these mice have a hypervascular phenotype progressing to multifocal hemangiomas (15). \textit{In situ} and RNase protection analyses show \textit{fps/fes} expression in vascular endothelial cells of the hemangiomas and in endothelial cells of normal blood vessels (15). Endothelial cell lines derived from the embryonic yolk sac of these 'MF' transgenic mice, such as the C166 cell line, have been found to have a growth advantage over endothelial cells that don't express the activated \textit{fps/fes} allele (31). In addition, the C166 cell line has a reduced requirement for a number of growth factors (31). Fps/Fes could potentially be acting downstream of a variety of vascular endothelial cell receptor tyrosine kinases, such as Flk-1, Flt-1, Flt-4, among others; but currently there is no evidence indicating a particular signal transduction pathway. In addition, results described in this thesis suggest that Fps/Fes activity is not required for vascular endothelial cell proliferation and blood vessel formation.

\textbf{Role in hematopoietic signaling pathways}

In hematopoietic cells, Fps/Fes becomes tyrosine phosphorylated and catalytically activated in response to granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) stimulation, suggesting Fps/Fes is acting downstream of the GM-CSF and IL-3 receptors (32,33). Indeed, GM-CSF appears to induce association between Fps/Fes and the β-chain of GM-CSF receptor (32,33). There is also evidence suggesting Fps/Fes is acting downstream of the erythropoietin and interleukin-4 receptors (34,35). Through association with these receptors and transduction of the
signal downstream, Fps/Fes may play a role in the differentiation of myeloid cells. High levels of Fps/Fes protein expression have been seen in mature granulocytes, monocytes and human myeloid leukemia cell lines, which can be induced to differentiate (36). It has also been shown that chicken myeloid stem cells infected by a retrovirus carrying the v-fps/fes oncogene differentiate \textit{in vitro} without the addition of exogenous growth factors (37). In addition, the K562 chronic myelogenous leukemia cells, in which $fps/fes$ expression is absent, are resistant to myeloid differentiation (38). Following transfection of these cells with an expression construct encoding the human Fps/Fes protein, clones expressing Fps/Fes and its associated kinase activity were able to undergo myeloid differentiation (38). Antisense experiments also suggest that Fps/Fes is required for the survival of granulocytes during myeloid differentiation, as cells treated with a specific $fps/fes$ antisense oligo undergo programmed cell death (39). Therefore, association of Fps/Fes with activated cytokine receptors allows the tyrosine phosphorylation and activation of Fps/Fes which appears to result in a signal towards differentiation of the myeloid cells. However, results described in this thesis suggest that Fps/Fes activity is not required for the differentiation of myeloid cells.

\textbf{Factors mediating Fps/Fes signaling}

Although a signal transduction pathway involving Fps/Fes has not been elucidated, some downstream factors have been implicated in mediating Fps/Fes signaling. \textit{In vitro}, tyrosine phosphorylated Fps/Fes has been shown to be able to associate with the SH2 domain of ras GTPase-Activating Protein (GAP); this association is thought to allow Fps/Fes to phosphorylate rasGAP (40). Since activated rasGAP enhances the GTP hydrolysis of p21ras and consequently may down-regulate the signal for proliferation,
activated Fps/Fes may signal myeloid cells to differentiate through its' interaction with rasGAP. Additional evidence to suggest Fps/Fes acts upstream of the ras pathway is that a dominant negative form of ras has been shown to prevent v-fps/fes transformation of NIH3T3 fibroblasts (41). Fps/Fes may also signal into the ras pathway by phosphorylating BCR on tyrosine and consequently inducing association of BCR with GRB-2/SOS, the ras guanine nucleotide exchange factor complex (21). Therefore, Fps/Fes may be inducing vascular endothelial cell proliferation by activating BCR which then activates the ras pathway thru GRB-2/SOS. Recently, one group has suggested that following GM-CSF stimulation a complex of JAK2, Fps/Fes, Stat1 and Stat3 associates with the GM-CSF receptor (33). How Fps/Fes is activated in this complex and exactly what proteins Fps/Fes phosphorylates are not clearly understood. Finally, tyrosine phosphorylation of 130 Kd and 75 Kd proteins has been seen in a macrophage cell line overexpressing c-fps/fes, and the SH2 domain of Fps/Fes was also shown to bind these phosphorylated proteins, suggesting that P130 and P75 are substrates of the Fps/Fes kinase (42). Although the identity and function of P75 is unknown, recently P130 has been identified as the same protein that is phosphorylated by Fyn in T cell activation (43). Cas, catenin-associated substrate, has also been identified as a substrate of Fps/Fes (43), suggesting Fps/Fes may play a role in cell adhesion and cell-cell interactions during immune responses of macrophages. In summary, Fps/Fes may be phosphorylating many different proteins in the cell; it may be associating with many different proteins, and Fps/Fes may even signal through the ras pathway (see figure 2). But in general, the downstream signaling pathways involving Fps/Fes are not clearly understood.
Fps/Fes is thought to be involved in signal transduction pathways in hematopoietic cells, and vascular endothelial cells, among other cell types. Association of Fps/Fes with activated cytokine receptors allows the tyrosine phosphorylation and activation of Fps/Fes. Fps/Fes may signal through the ras pathway by associating with rasGAP or BCR, but many other factors have been found associated with Fps/Fes. These signal transduction pathways are not clearly understood. Evidence suggests Fps/Fes is involved in the signal resulting in the differentiation of hematopoietic cells and involved in the signal resulting in the proliferation of vascular endothelial cells. Depending on what substrates of Fps/Fes are present in a given cell at a given time, activation of Fps/Fes may result differentiation, proliferation, or other cellular functions. Recent evidence suggests Fps/Fes may be involved in secretion or endocytic type processes and cell adhesion or cell-cell interaction type processes.
Hematopoietic cell

Vascular endothelial cell

(tL-3 or GM-CSF

receptor

Ras\:GDP

SOS

Grb2

Ras\:GTP (active)

rasGAP

Frz/Fes

Jaks

Sirt1

complex

differentiation

or other cellular functions

(proliferation

(secretion, cell adhesion or cell-cell interaction)

VEGF

receptor
Research suggests that Fps/Fes may be involved in the signal transduction pathways resulting in vascular endothelial cell proliferation and myeloid cell differentiation. Depending on what substrates of Fps/Fes are present in a given cell at a given time, activation of Fps/Fes may result in proliferation or differentiation of that cell. In addition, since Fps/Fes has been found to localize to the perinuclear region in cultured neuronal, epithelial and endothelial cell lines, and this appears to be coincident with the Golgi apparatus (14), then Fps/Fes may also play a role in secretion in a variety of cell types, such as Leydig cells, chondrocytes, neurons, and glandular cells of the uterus. More recently, the identification of Cas as a substrate of Fps/Fes in macrophages (43) suggests a role for Fps/Fes in cell adhesion, possibly in a variety of cell types. The molecular mechanisms controlling hematopoiesis, blood vessel formation, secretion and cell adhesion in different cell types are not clearly understood. The involvement of Fps/Fes in these processes is just beginning to be unraveled with the phenotypic analysis of transgenic mice expressing a “loss of function” mutation in the fps/fes gene.

Hematopoiesis

Hematopoiesis is simply defined as the differentiation and maturation of stem cells, which gives rise to all mature blood cells. This process can be divided into lymphopoiesis, which gives rise to B and T lymphocytes, and myelopoiesis, which gives rise to all the other blood cells (see figure 3). Hematopoiesis involves not only the generation, proliferation and maintenance of stem cells but also the lineage commitment and maturation of immature progenitor cells. Hematopoiesis first occurs in the blood islands of the yolk sac, then in the liver at midgestation, and just before birth the bone
Hematopoiesis is the differentiation of pluripotential and multipotential stem cells into mature lymphoid and myeloid cell types. The mature lymphoid cell types are the B and T lymphocytes. The mature myeloid cell types are the erythrocyte, platelets, monocyte, and the granulocytes (neutrophils, eosinophils and basophils). The microenvironment and the presence of growth factors, such as colony-stimulating factors and cytokines play key roles in hematopoiesis. This figure was adapted from the textbook “Basic Histology” by L. C. Junqueira, J. Carneiro, and R. O. Kelley (44).
Lymphoid multipotent cells

Myeloid multipotent cells

Pluripotent cell

B and T lymphocyte

erythrocyte

platelets

monocyte

neutrophil

eosinophil

basophil
marrow becomes the major site of hematopoiesis. Hematopoiesis depends on the presence of a suitable microenvironment, which is provided by the extracellular matrix of a hematopoietic organ, and the presence of growth factors, such as colony-stimulating factors or cytokines, which stimulate proliferation and/or differentiation of immature cells. Hematopoiesis can be mimicked in vitro with the use of methylcellulose, a semisolid tissue culture medium, which provides a suitable microenvironment, and with the addition of appropriate growth factors (45,46); methylcellulose assays can be useful in determining the molecular mechanisms involved in blood cell development. This assay has been recently used to show that the SCL/tal-1 transcription factor is required for the generation of all the mature hematopoietic cells; consequently, SCL/tal-1 is thought to be involved in the induction of mesodermal cells or in the generation or maintenance of immature progenitors (47). Whether or not Fps/Fes is required for the generation of mature hematopoietic cells has been investigated with the use of methylcellulose assays, and is described in this thesis.

**Vasculogenesis and Angiogenesis**

During embryonic development, at least two different processes appear to be involved in the formation of blood vessels. 'Vasculogenesis' is the development of blood vessels from *in situ* differentiation of angioblasts or primitive endothelial cells; whereas, 'angiogenesis' is the sprouting of blood vessels from preexisting vessels (see figure 4) (for a review see (48)).

The molecular mechanisms controlling vasculogenesis and angiogenesis are poorly understood. Many factors may play a role in regulating blood vessel formation including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and
Figure 4: Blood vessel formation: vasculogenesis and angiogenesis.

This is an illustration of the steps involved in blood vessel formation during embryonic development. Vasculogenesis, the in situ differentiation of angioblasts, is illustrated in four steps. Fibroblast growth factor (FGF), secreted from endodermal cells, is thought to stimulate mesodermal cells to differentiate into blood islands. The fusion of blood islands and endothelial cell differentiation allows the formation of a primary capillary. Angiogenesis, the proliferation and migration of endothelial cells from existing vessels, allows complex capillary networks to be formed. This figure was adapted from "Vasculogenesis" by W. Risau and I. Flamme (48).
1. Mesoderm formation
   migrating mesodermal cells
   → ectoderm
   → endoderm

2. Blood island differentiation
   angioblasts
   → hematopoietic cells
   → blood islands

Vasculogenesis

3. Fusion of blood islands and endothelial cell differentiation
   extracellular matrix
   → lumen
   → endothelial cells

4. Primary capillary

Angiogenesis
   (proliferation and migration of endothelial cells from existing vessels)
platelet-derived growth factor (PDGF). Receptors for these growth factors such as the VEGF receptors: Flk-1, Flt-1, and Flt-4, as well as nonreceptor tyrosine kinases appear to play a role in the proliferation and differentiation of endothelial cells and the formation of blood vessels. The expression patterns of the VEGF receptors give an indication of their possible function; for example, Flk-1 is the first VEGF receptor expressed in development (embryonic day 7.0) and consequently it could be predicted that it is involved in the early determination of the endothelial lineage. But the knockout phenotype gives a better indication of receptor function; for example, the knockout of \textit{flk-1} suggested that Flk-1 is required for blood island formation (49). The absence of Flk-1 signaling impaired endothelial cell formation, where as the absence of Flt-1 signaling led to abnormal assembly of the endothelial cells, i.e. abnormal vascular channels (50). As for the Tie-2 receptor tyrosine kinase, it appears to be important in angiogenic processes since Tie-2 \textit{-/-} embryos are growth retarded and lack smaller blood vessel branches as shown in the heart and brain regions (51). The Tie-1 receptor appears to play a role in establishing blood vessel integrity, as hemorrhaging was seen throughout the embryo (51). The knockout phenotypes of all these receptor tyrosine kinases suggests that these receptors have distinct functions in the process of blood vessel formation. Elucidating the signal transduction pathways from these receptors will help decipher the molecular mechanisms involved in vasculogenesis and angiogenesis. Fps/Fes may be acting downstream of any of these receptors. It is interesting that Songyang et al. have found that the SH2 domain of Fps/Fes may potentially bind to a phospho-tyrosine motif in the Tie-2 receptor tyrosine kinase (27). However, results described in this thesis suggest that Fps/Fes activity is not essential in blood vessel formation: vasculogenesis or angiogenesis.
**Fer**

P94 Fer was first identified using anti-fps serum that cross-reacted with a 94,000-dalton protein (6,52). Fer, which stands for "fps/fes-related", was found to have tyrosine kinase activity and a wide cell type and tissue distribution (6,52,53,54, 55). In particular, P94 Fer was found in a number of human or mouse myeloid, erythroid, and lymphoid cell lines (6,54), as well as in a number of mouse and rat tissues (6,53). In chickens, P94 levels were highest during embryonic development and in the adult gizzard, brain and spleen (52). The ubiquitous expression of P94 suggests that this protein may be involved in an essential function of all cells. In 1990, a cDNA corresponding to an alternatively spliced form of the fer mRNA was isolated from a mouse testis cDNA library and was called “ferT”; the encoded 51,000-dalton protein includes the SH2 and kinase domains of P94 Fer but has a different N-terminal domain (56). In contrast to P94Fer, FerT was found to be exclusively expressed in the testis, predominantly in primary spermatocytes, which is a specific stage of germ cell development, and thus FerT is thought to be involved in regulating a distinct step of spermatogenesis (56,57).

**Knockouts**

Generating transgenic mice that have a gene 'knocked-out' or a gene that encodes a nonfunctional product can give great insight into the biological function of that gene; 'knockouts' address the normal role of a gene in development and determine whether or not it is required for particular processes. However, the function of a gene may be masked in some 'knockouts' due to functional redundancy; if the protein is not produced as in a 'null' knockout, it has been found that other genes may compensate for that loss.
and the resulting phenotype is normal. For example, although c-src has been found to be widely expressed, the phenotype of the src knockout mouse is very restricted. The src knockout mouse displayed osteopetrosis due to a defect in bone remodeling by osteoclasts; but there were no obvious abnormalities in the brain or platelets, where c-src is most highly expressed (58). This suggested that either Src is not essential in the central nervous system and platelets or other src family members, which are also widely expressed, are compensating for the loss of Src in the src knockout mouse. A knockout of one of the other src family members, c-fyn, displayed no overt phenotype; upon closer examination defects in T cell receptor signaling and neurological abnormalities were found (59,60). When the src knockout mouse was crossed with the fyn knockout mouse the phenotype was embryonic lethal; this phenotype is more severe than the sum of the two individual knockout phenotypes and consequently suggested that Fyn was compensating for the loss of Src in the src knockout mouse (61).

**Experimental Rationale**

It is possible that Fer, the most closely related cytoplasmic tyrosine kinase to Fps/Fes, or another yet unknown cytoplasmic tyrosine kinase may compensate for the loss of Fps/Fes in a 'null' knockout. Using a strategy of 'knocking-out' the fps/fes proto-oncogene by creating a "loss of function" mutation, such as a lysine to arginine amino acid substitution which has been shown to abolish the catalytic activity of tyrosine kinases (described in detail on page 52 of this thesis), maintains normal Fps/Fes protein levels. Consequently, functional redundancy is less likely to occur, as the mutant Fps/Fes protein will still be able to interact with upstream and downstream substrates in the cell (see figure 5). The role Fps/Fes plays in blood vessel formation,
Figure 5: Illustration of the concept of functional redundancy.

If Fps/Fes is absent in a cell then a closely related protein, such as Fer, may compensate for its loss by performing its function, and consequently no abnormality or phenotype is seen due to the loss of Fps/Fes. If Fps/Fes is absent in the cell, then Fer may associate with receptors for which active Fps (shown as Fps-K) would normally associate. Fer may then be able to transduce the signal downstream, and consequently, the cell still receives the cellular proliferation and/or differentiation signal. Furthermore, the cell appears to be functioning normally and no phenotype is seen due to the loss of Fps/Fes. Using a strategy of 'knocking-out' the fps/fes proto-oncogene by creating a "loss of function" mutation, shown as Fps-R in the diagram, maintains normal Fps/Fes protein levels; consequently, functional redundancy is less likely to occur. Thus a phenotype due to the catalytically inactivated Fps/Fes protein (Fps-R) is more likely to be seen, compared to a "null" knockout where the protein would be absent.
functional redundancy

cellular proliferation and/or differentiation

Fps/Fes

K

R

OH

Fer

?
hematopoiesis, and secretion in a variety of cell types has been addressed in the analysis of transgenic mice expressing a nonfunctional Fps/Fes kinase.

This thesis illustrates the generation and characterization of rabbit polyclonal antisera raised against various regions of the Fps/Fes and Fer proteins so that characterization of the "loss of function" Fps/Fes mutation could be accomplished. The generation of the mutation and the verification that it abolished the catalytic activity of the Fps/Fes protein in vitro is then demonstrated. Finally, the thesis illustrates the generation and characterization of transgenic mice expressing a targeted inactivating ("loss of function") mutation in the fps/fes gene, divided into the following parts: a) generation of a targeting construct to introduce the mutation into embryonic stem (ES) cells, b) isolation of targeted ES cell clones with the point mutation, c) generation of chimeric mice and breeding of the mice to establish stable transgenic lines (heterozygotes and homozygotes for K to R mutation in Fps/Fes), and d) analysis of the biological phenotype.
CHAPTER 2: MATERIALS AND METHODS

Generation of rabbit polyclonal antisera

Since the anti-Fps Q381-E566 serum (previously generated by P. Greer) was found to be cross-reactive to both Fps/Fes and Fer proteins (like many of the anti-Fps antisera used in the literature (52)), a number of polyclonal anti-sera raised against various regions of the Fps/Fes or Fer proteins were generated (see figure 6). The anti-Fps Q381-E566 serum was raised against the region of the murine Fps/Fes protein from glutamine 381 to glutamic acid 566. Since there is 54% identity in this region between the Fps/Fes and Fer proteins and the anti-Fps Q381-E566 serum was found to cross-react with Fer, new polyclonal antisera were raised against regions of Fps/Fes or Fer with a lower percent identity. The polyclonal serum anti-Fer L97-A382 was raised against the region of the murine Fer protein from leucine 97 to alanine 382; whereas, the polyclonal serum anti-Fps L102-Q378 was raised against the region of the murine Fps/Fes protein from leucine 102 to glutamine 378. There is 36% identity in this region between the Fps/Fes and Fer proteins. The anti-Fps V306-Q378 serum was raised against the region of the murine Fps/Fes protein corresponding to valine 306 to glutamine 378. The anti-Fer L305-A378 serum was raised against the region of the murine Fer protein corresponding to leucine 305 to alanine 378. There is 27% identity in this region of the Fps/Fes and Fer proteins; this region was selected with the aim of obtaining antisera that would detect Fps/Fes or Fer specifically. The GST-fusion proteins, containing a seven amino acid histidine track, were expressed in Escherichia coli bacteria by induction with IPTG (isopropyl β-D-thiogalactopyranoside; Sigma) and purified on a nickel-chelate column as directed by the manufacturer (Qiagen; detailed
Figure 6: GST-fusion proteins used to generate the polyclonal antisera.

GST-fusion proteins containing various regions of the Fps/Fes or Fer proteins are illustrated. GST-Fps Q381-E566 contained the SH2 domain, as well as some of the kinase and amino-terminal domains of Fps/Fes; but the polyclonal antiserum obtained was found to be cross-reactive to the Fer protein. Since the lowest sequence identity was found in the N-terminal domains of Fps/Fes and Fer, the other GST-fusion proteins contained different portions of this domain, in hopes that more specific antisera could be generated. These GST-fusion proteins were expressed in Escherichia coli and then purified on a nickel-chelate column with the use of the seven amino acid histidine track (H). The purified proteins were then used to immunize rabbits.
FPS / FER

N ── SH2 ── KINASE ── C

N ── GST ── Fps ── H ── C

Q381 E566

N ── GST ── Fps ── H ── C

V306 Q378

N ── GST ── Fps ── H ── C

L102 Q378

N ── GST ── Fer ── H ── C

L97 A382

N ── GST ── Fer ── H ── C

L305 A378
under ‘Induction and purification of GST-fusion proteins’). The purified proteins were then used to immunize rabbits to generate the polyclonal sera.

**Generation of GST-fusion proteins**

The PCR products of the designated regions of *fps/fes* or *fer* were cloned into the pGEX-His vector, which was produced from pGEX-2T (62) by inserting an oligo encoding the (His)$_9$ tract into the EcoRI site (P. Greer, unpublished), using BamHI and EcoRI sites. Sequencing confirmed that the fusion proteins were in frame. Oligos to PCR clone murine Fps/Fes cDNA encoding valine 306 to glutamine 378 from pBLTN-4 (gift from Andrew Wilks)(10) into the pGEX-His vector were MFesNV306: 5'-AAC GGA TCC GTG CAA CAC ACG CTG-3' and MFesCQ378: 5'-C CTG AAT TCC CTG AAG CTT GTC CTG-3'.

PCR oligos to amplify and clone murine Fer cDNA encoding amino acid sequences between L-305 to A-378 from a “Friend” erythroleukemia cDNA library (gift from Yacov Ben David) into the pGEX-His expression plasmid were MFerNL305: 5'-GCA GGA TCC TTG CAA GTC ATG TG-3' and MFerCA378: 5'-C TTG AAT TCC TGC ACA CTT TGC CTC-3'.

Oligos used to PCR clone murine Fer cDNA encoding leucine 97 to alanine 378 from PECE-Fer, a murine fer cDNA construct (gift from Ken Letwin), into the pGEX-His vector were MFerNL97: 5'-TCT GGA TCC TTG CAC AGG CTC ACC-3' and MFerCA382: 5'-CTC GGA TCC AGC TTT CTG TGC TGC-3'. Oligos to PCR clone murine Fps/Fes cDNA encoding leucine 102 to glutamine 378 from pBLTN-4 were MFesNL102: 5'-CCC TGA ATT CAA CTG AGC GTG CTG ATC-3' and MFesCQ378: 5'-C CTG AAT TCC CTG AAG CTT GTC CTG-3'.
**Induction and purification of GST-fusion proteins**

A 25 ml overnight culture of pGEX-fps or pGEX-fer transformed Escherichia coli was innoculated into a 500 ml culture and incubated for 2 hrs at 37°C. To induce expression of the GST-fusion proteins, IPTG was added to 1mM and the culture was incubated for 6 hrs at 37°C. The inductions were verified by electrophoresis of the total bacterial proteins from an aliquot of the culture on an 11% polyacrylamide gel and then staining the gel with Coomassie blue (Sigma).

To isolate the GST-fusion proteins, the cells were suspended in lysis buffer A (6M guanidine-HCl/0.1M NaH$_2$PO$_4$/0.01M Tris pH 8.0/10 mM β-mercaptoethanol) 5 mls per 1 gram of cells, and then incubated on a rotator platform at room temperature for 1hr. Following centrifugation at 10000rpm for 15 minutes at 4°C to remove cellular debris, the supernatant was applied to the nickel-chelate column (Qiagen) equilibrated in lysis buffer A. The column was washed once with 10 mls buffer B (8M urea/0.1M NaH$_2$PO$_4$/0.01M Tris/10 mM β-mercaptoethanol pH 8.0), and then once with 10 mls buffer C (8M urea/0.1M NaH$_2$PO$_4$/0.01M Tris/10 mM β-mercaptoethanol pH 6.3). The GST-fusion proteins were eluted in four 5 ml fractions of buffer D (8M urea/0.1M NaH$_2$PO$_4$/0.01M Tris/10 mM β-mercaptoethanol pH 5.9), followed by two 5 ml fractions of buffer E (8M urea/0.1M NaH$_2$PO$_4$/0.01M Tris/10 mM β-mercaptoethanol pH 4.5). Aliquots of the eluted fractions were electrophoresed on an 11% polyacrylamide gel, and those fractions containing the GST-fusion protein were combined and dialyzed against 50 mM NaHCO$_3$ (BDH). The amount of purified GST-fusion protein was then quantitated on an 11% polyacrylamide, Coomassie blue stained gel using BSA controls.
Immunization of rabbits and collection of antisera

Antisera were raised in New Zealand white rabbits (Charles River Laboratories). After taking preimmune blood by ear vein bleeding, 1 mg of purified GST-fusion protein mixed as a 50/50 emulsion with complete Freund adjuvant (DIFCO Laboratories) was injected intramuscularly and subcutaneously into the rabbit by Dr. Peter Greer. One month after injection, the rabbit was boosted using 100 μg purified GST-fusion protein mixed with incomplete Freund adjuvant (DIFCO Laboratories) and bled 10 days after the boost. This was repeated a number of times until the antisera obtained Western blotted or immunoprecipitated the desired Fps/Fes or Fer protein.

Approximately 30 - 40 mls of blood was collected from each rabbit. Clotting was promoted by incubation at 37°C for one hour, then allowed to proceed at 4°C O/N. Following centrifugation at 2000 rpm for 15 minutes at 4°C (Sorval), the serum was collected and 0.05% sodium azide was then added. The antiserum was aliquotted and stored at -80°C.

Affinity purification of polyclonal antisera

To affinity purify the polyclonal antisera, initially columns of the GST-fusion protein bound to cyanogen bromide activated sepharose beads (Sigma) needed to be made. To make the columns, inductions of GST-Fps L102-Q378 and GST-Fer L97-A382 were performed as described above, and then the GST-fusion proteins were solubilized as described by Frangioni and Neel (63). The soluble GST-fusion protein was dialyzed against the Coupling Buffer (0.1M NaHCO₃ pH8.3, 0.5M NaCl) and then bound to cyanogen bromide activated sepharose beads (Sigma). These beads were washed with Coupling Buffer then Sodium Acetate Buffer (0.1M sodium acetate pH4, 0.5M NaCl)
three times, then washed with Coupling Buffer, followed by a PBS wash. The beads were then suspended in PBS and poured into disposable Bio-Rad column. The column was stored in PBS with 0.5% sodium azide at 4°C.

To affinity purify the anti-Fps V306-Q378 serum for example, a 3 ml aliquot of anti-Fps V306-Q378 serum was initially cycled through the GST-Fps column at 4°C O/N. The column was washed with PBS, then 2M KCl-PBS. The anti-Fps antibodies were eluted with NaI/sodium thiosulfate buffer (5M NaI/1mM Na₂S₂O₃ made up fresh) in eight 1 ml fractions. To re-equilibrate the column, it was washed with PBS, then HCl-Glycine (0.2M Glycine pH2.2), and then PBS. To determine which fractions contained the antibodies, spectrophotometer readings at OD290 were taken. Since proteins are not stable in NaI/Nalithiosulfate buffer, these fractions were then pooled and run over a DG-10 column (Bio-Rad) to change to TBS buffer (20mM Tris-HCl pH8 (BDH), 150mM NaCl (Fisher)). To stabilize the antibodies, BSA was added to 10 mg/ml final concentration in TBS. This partially affinity purified Fps V306-Q378 was cycled through the GST-Fer column; the flow-through from this heterologous column was collected and called the affinity purified antibody. The anti-Fer L305-A378 serum was then affinity purified analogous to the method described above for the affinity purification of the anti-Fps V306-Q378 serum.

**Cos-1 cell culture and transfection**

Cos-1 cells (monkey kidney cell line)(64) were grown in Dulbecco's modified Eagle's medium (DMEM)(Gibco BRL) with 10% fetal bovine serum (Hyclone) at 37°C and 5% CO₂ on tissue culture plates. To passage the cells, 1.5 ml of trypsin EDTA (Difco) was added to the plate, and incubated for approximately 1 minute at room
temperature. Cos-1 cells were subcultured such that they were 25-50% confluent on day of the transfection in 35mm tissue culture dishes (6 well plate). Each transfection was performed as follows. One or two micrograms of DNA was added to 100 μl OptiMEM (Gibco BRL). This was mixed with 5 μl lipofectamine (Gibco BRL) in 100 μl OptiMEM and then incubated at rt for 1 hour. OptiMEM was added up to 1 ml and this mixture was then added to cells prewashed with PBS and OptiMEM. After a five hour incubation at 37°C, 5% CO₂, 2 mls of DMEM/10% FBS was added to each well. The Cos-1 cells were harvested 48 hours later.

Harvesting Cos-1 cells

After washing the transfected Cos-1 cells twice with cold TBS, the cells were lysed in kinase lysis buffer (20mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA (BDH), 1% NP40 (BDH), 0.5% sodium deoxycholate (Sigma), with freshly added 10 μg/ml leupeptin (Sigma), 10 μg/ml aprotinin (Sigma), 1mM PMSF (Gibco BRL), 1mM Na₃VO₄ (Aldrich).

For whole cell lysate samples, equal volume of 2X SDS sample buffer (10mM sodium phosphate pH6.8 (BDH), 4% SDS (BDH), 0.2M DTT (ICN), 10% β-mercaptoethanol (Sigma), 20% glycerol (ICN), 0.4% bromophenol blue (Sigma)) was added to the clarified lysate.

For immunoprecipitations, 20 μl 50% Protein A sepharose beads (Sigma) and 5 μl antiserum (anti-GST-Fps Q381-E566, preimmune Fps Q381-E566, anti-GST-Fer L97-A382 or preimmune Fer L97-A382) were added to the clarified lysate. After rocking 2-4 hours 4°C, the beads/immunoprecipitates were washed four times in KLB (kinase lysis buffer). Addition of 2X SDS sample buffer and a 20 minute incubation at 37°C
allowed the beads to be dissociated from the immune complexes. Samples were heated 3-5 minutes at 100°C before resolving the proteins by SDS-PAGE; 7.5% acrylamide gels were used to resolve proteins in the 90 Kd region, such as P92 Fps and P94 Fer.

Western blotting

After resolving the proteins by SDS-PAGE, they were then transferred to Immobilon-P membrane (Millipore) in Western transfer buffer (190 mM Glycine (ICN), 25 mM Tris-HCl pH 8.0, 0.07% SDS, 20% methanol) for 1 hour using a semidy transfer apparatus (Bio-Rad). Membranes were blocked overnight at 4°C with BLOTTO (5% Carnation skim milk powder in TBS-T (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.1% Tween-20 (Sigma)). The rabbit polyclonal antiserum, which was diluted in BLOTTO at 1:100 to 1:1000 for crude antiserum or at 1:5 dilution for the affinity purified antiserum, was used as the primary antibody and incubated with the membrane at room temperature for two to four hours. After washing the membranes for 15 minutes and then twice for 5 minutes with TBS-T, the membranes were incubated with a 1:16000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham or Vector Laboratories) in TBS-T for 1 hour at room temperature. After washing for 15 minutes with TBS-T and then four times 5 minutes with TBS-T, the immune complexes were detected using enhanced chemiluminescence (DuPont NEN). The membranes were stripped using Western stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 7.0), for 30 min. at 50°C, then washed with TBS-T and blocked in BLOTTO O/N at 4°C.
Preparation of mouse tissue lysates

Tissues were dissected from the mice and put in 15ml conical tubes on ice. The tissue was homogenized in 5 mls PLC lysis buffer (50 mM HEPES (Fisher)pH 7.0, 150 mM NaCl (Fisher), 10% glycerol (ICN), 1% Triton X-100 (ICN), 1.5 mM MgCl₂ (Fisher), 1 mM EGTA (Sigma), 100 mM NaF (BDH), 10 mM disodium pyrophosphate (Sigma), in which 10 µg/ml aprotinin (Sigma), 10 µg/ml leupeptin (Sigma), 1 mM PMSF (Gibco BRL), 1mM Na₃VO₄ (Aldrich) was added fresh. Aliquots of the tissue lysate were centrifuged to remove cellular debris, and 2X SDS sample buffer was added to the clarified lysate in a 1:1 ratio. Total tissue protein loaded on SDS-PAGE gel was quantitated by Coomassie blue staining.

Immune complex kinase assays

Cos-1 cells or mouse bone marrow cells, flushed from the femurs of 6 week old mice, were lysed in kinase lysis buffer, and Fps/Fes and Fer proteins were immunoprecipitated as described above. After washing the immune complexes five times in KLB, the immune complexes were washed once in kinase reaction buffer (KRB)(20 mM TrisHCl pH 7.5, 10 mM MnCl₂ (Sigma), 100 µM Na₃VO₄). The immunoprecipitates were then incubated with 10 µCi γ²³²P-ATP (Dupont NEN) in KRB at room temperature or 37°C for 20 minutes. The addition of 2X SDS sample buffer and a 20 minute incubation at 37°C allowed the immune complexes to be dissociated from the beads. Proteins were resolved by SDS-PAGE, and then Western blotted to quantitate Fps/Fes and Fer proteins in the immune complexes. In addition to Western blotting, after resolving the proteins by SDS-PAGE, the gel was fixed in 10% glacial acetic acid (Fisher), 20% methanol, 100 mM sodium pyrophosphate (Sigma) and exposed to Kodak
X-ray film (Mandel) to look at tyrosine kinase/autophosphorylation activity. These assays were also performed using enolase (Sigma) as an exogenously added substrate.

**Generation of expression constructs and PCR mutagenesis**

The murine *fps/fes* locus was cloned from a genomic library, prepared from DNA isolated from a 129/Sv mouse, by Ralph Zirngibl (see figure 7, panel A). Standard molecular biology cloning techniques were used to generate expression constructs encoding either the wild type or mutant Fps/Fes kinase. Although this was completed as part of my fourth year thesis project, these constructs were used in my Masters thesis project and for completeness it is important to describe them here. To generate the wild type expression construct, initially the XbaI restriction site in the SV40 based expression plasmid PECE (65) was converted to NotI site using an adaptor linker; this plasmid was called PXN. The 15 Kb NotI fragment of *fps/fes* (from the plasmid *pfesgen11A* provided by Ralph Zirngibl), which included the complete *fps/fes* locus, was subcloned into PXN#1; this vector is the wild type expression plasmid, called PXNK (see figure 7, panel B). Subclone PXNK#4 was determined to be the positive orientation of the *fps/fes* gene, relative to the SV40 early promoter (PE) in PECE.

To generate the expression plasmid encoding the mutant Fps/Fes kinase, initially the mutation, an adenine (A) nucleotide converted to a guanine (G) nucleotide, needed to be generated by PCR mutagenesis. An XbaI fragment isolated from pmfes1NK11A (3.5 Kb Kpnl fragment of *fps/fes* in pBluescriptII KS from Ralph Zirngibl) was subcloned into pGEM-1 vector; this was used as a template in the PCR. The mutagenic oligos were sense GTG GCT GTG AGA TCT TGC CGA and anti sense TCG GCA AGA TCT CAC AGC CAC. Four polymerase chain reactions were set up using four combinations of primers, mutagenic
Figure 7: Generation of expression and targeting constructs.

The murine fps/fes locus, illustrated in panel A, was cloned from a genomic library, prepared from DNA isolated from a 129/Sv mouse, by Ralph Zirngibl. Some of the mapped exons are shown as black boxes, and the restrictions sites significant in cloning the expression and targeting vectors are shown. To generate the wild type expression plasmid (PXNK4), shown in panel B, the 15 Kb NotI fragment from pfesgen11A was subcloned into a modified PECE expression plasmid. To generate the lysine to arginine mutation, PCR mutagenesis using the Xba fragment, shown in panel B, was performed. To generate the mutant expression plasmid (PXNR24) shown in panel C, an Xba fragment containing the mutation, which was derived from the PCR mutagenesis, was substituted into PXNK4. To generate the targeting vector (9KbRKS-pPNT450NHS#17) shown in panel D, initially the 450 bp EcoRI fragment was subcloned into a modified pPNT vector, such that it was between the neomycin resistance cassette and the herpes simplex thymidine kinase gene. The 9 Kb EcoRI fragment from PXNR24 was subsequently subcloned into the modified pPNT vector containing the 450 bp EcoRI fragment. For details of the subcloning see the Materials and Methods.
A.  \textit{pfesgen11A} (murine \textit{fps/fes} genomic locus)

B.  \textit{PXNK4}

C.  \textit{PXNR24}

D.  \textit{9KbRKS-pPNT450NHS#17}
sense or anti sense with the T7 primer (21 mer) or the reverse primer (16mer). Using pfu polymerase, the PCR conditions for one cycle were one minute at 95°C, then one minute at 50°C, and five minutes at 72°C. After 25 cycles, 10 minutes at 72°C and holding at 4°C completed the reaction. The PCR products obtained from two of these reactions were purified using a primerase column (Stratagene) and then combined to be used in another polymerase chain reaction. This reaction, using the T7 and reverse primers, generated the full length Xbal fragment containing the mutation. This Xbal fragment was then subcloned into pGEM-1 (Promega) and called pGXR1. The mutation was confirmed by sequencing as per the manufacturers instructions (Pharmacia T7 Sequencing kit). Finally, the Xbal fragment from pGXR-1 was substituted into PXNK4. This plasmid was called PXNR and subclone PXNR#24 was found to be the positive orientation (see figure 7, panel C). This mutation generated a novel BglIII restriction site which was used in subsequent experiments. In addition, this mutation resulted in a lysine (K) residue, critical for kinase activity, being converted to an arginine (R) residue at the protein level.

**Generation of targeting vector**

To generate a targeting vector to introduce this lysine to arginine mutation, the "loss of function" mutation into embryonic stem (ES) cells, the 9 Kb EcoRI fragment from PXNR24 was initially subcloned into pBluescriptII KS-. Subclone #8 (9KbRlIpBSKS-#8) was then cut with NotI and Sall to excise the 9 Kb fps/fes fragment, and directionally subcloned into pPNT450NHS #14.2. The pPNT450NHS #14.2 was a modified pPNT vector (66). To generate the pPNT450NHS #14.2 initially, a NotI/Hpal/Sall linker was subcloned into the XhoI site of pPNT by Ralph Zirngibl, such
that the linker was 5' of the neomycin resistance cassette. The 3' 450 bp EcoRI fragment of murine \textit{fps/fes} was subsequently subcloned into the EcoRI site of pPNT450NHS, such that the 450 bp EcoRI fragment was between the neomycin resistance cassette, the positive selection cassette, and the herpes simplex thymidine kinase gene, the negative selection cassette. Consequently, the targeting vector consisted of a 9 kb long arm of homology, a neo cassette needed for positive selection, a 450 bp short region of homology in the 3' end of \textit{fps/fes}, and a tk cassette used for negative selection (see figure 7, panel D). Details of the targeting strategy are described in the results section of this thesis. The targeting vector was called 9KbRKS-pPNT450NHS#17. This construct was maxiprepped with the use of cesium chloride (CsCl)(ICN) gradient centrifugation (as described in Current Protocols in Molecular Biology), and then linearized with NotI.

**ES cell culture and electroporation**

The embryonic stem (ES) cell line RI (obtained from Andras Nagy) is a pluripotent ES cell line shown to generate completely cell cultured-derived mice (67). ESRI cell line was generated from mating 129/Sv strain of mouse with 129/SvJ strain of mouse; thus, this ES cell line has agouti coat colour alleles and eye-pigmentation marker alleles, which are dominant over CD1 coat colour and eye-pigmentation alleles (67). ES cells were cultured on tissue culture plates coated with 0.1% gelatin (Sigma) or on embryonic fibroblasts, essentially as described by A. L. Joyner (68). The ES cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL) supplemented with 15% fetal bovine serum (Immunocorp), 2 mM glutamine (Gibco BRL), 10^{-6} M 2-mercaptoethanol (Sigma),1mM MEM sodium pyruvate (Gibco BRL), 0.1 mM MEM non-
essential amino acids (Gibco BRL) and 3 ng/ml recombinant leukemia inhibitory factor (LIF) at 37°C and 5% CO₂, and the media was changed every day. To freeze down ES cells, they were washed with PBS, trypsinized, diluted with complete media, centrifuged at 700 rpm for 5 minutes and resuspended in 0.9 mls DMEM. This 0.9 mls was added to 0.9 mls of 2X freezing media (40%DMEM, 40%FBS, 20%DMSO (Sigma)) that had been aliquotted to cryovials. The cryovials were put in styrofoam, inverted once and put at -70°C for at least 24 hrs; then they were transferred to liquid nitrogen for long-term storage.

To prepare the ES cells for electroporation, they were washed with PBS, trypsinized, centrifuged at 700 rpm for 5 minutes and preplated for 30 minutes at 37°C. The nonadherents/undifferentiated ES cells were then collected and centrifuged as above. These cells were resuspended in 0.8 mls cold PBS, and 50µg of linearized targeting vector in 50µl filtered PBS was added. The cells and DNA were transferred to a cuvette (Biorad) and electroporated with a Biorad gene pulser set at 240V and 500µF. Following a 20 minute incubation on ice, the ES cells were resuspended in 40 mls media (described above) and plated on four gelatinized 10 cm tissue culture plates. Selection was started two days following the electroporation by the addition of 200µg/ml G418 (Gibco BRL), a neomycin analog, and 2 µM Gancyclovir (GANC) (Syntex). The ES cells were cultured in media containing 200µg/ml G418 and 2 µM Gancyclovir for 6-8 days, until good size colonies appeared.
Screening ES cell clones

The G418 and GANC resistant ES cell clones were screened for homologous recombinants, essentially as described by W. Wurst and A. L. Joyner (68). The G418 and GANC resistant colonies were initially picked and seeded onto gelatin-coated 24 well tissue culture plate. This was performed by rinsing the 10cm plate with PBS, adding 1.5ml trypsin and aspirating; then the cells of each colony were scraped free using a pipette tip and collected with 200μl media, and expelled into individual wells of a 24-well cluster dish. After approximately 4 days, when the colonies had grown up in the 24-well dishes, the ES cell clones were trypsinized and resuspended in 1 ml media; 100μl of this 1ml was transferred to new 24-well dishes and another 100μl was transferred to an eppendorf tube for PCR analysis. Initially, the PCR analysis was done on pools of six ES cell clones. After the ES cells were pelleted, 40μl ddH2O was added and this was transferred to PCR tubes. The tubes were put on dry ice for 10 minutes and paraffin oil (BDH) was added. The tubes were heated at 95°C for 10 minutes, and cooled to 50°C so that 1μl of 10 mg/ml Proteinase K (BDH) could then be added. The Proteinase K was allowed to act for 90 minutes at 50°C, and then it was heat inactivated by incubation for 25 minutes at 95°C. PCR was completed in a 60μl reaction containing 43.7μl ddH2O, 10μl 10X Taq PCR buffer (Gibco BRL), 3μl 50mM MgCl₂ (Gibco BRL), 0.2μl 100mM dATP (Pharmacia), 0.2μl 100mM dCTP (Pharmacia), 0.2μl 100mM dGTP (Pharmacia), 0.2μl 100mM dTTP (Pharmacia), 1μl of the downstream primer mfs1.6RC#2 (5'-GACAGGGTTTCCGTATGTG-3') (515ng/μl), 1μl of the upstream primer neo21 (5'-CCGCTTCTCGTCTTACCGG-3') (984 ng/μl), and 0.5μl Taq polymerase (Gibco BRL). PCR conditions were as follows: a 'hot start' at 95°C, 40 cycles (95°C for 1 min., 52°C for 1 min. and 72°C for 3 min.), 72°C for 10 min., and
4°C hold. The PCR products were resolved on a 1% regular agarose gel (Gibco BRL), and a band of approx. 1 Kb could be routinely seen by ethidium bromide (EtBr)(Sigma) staining in some of the pools of ES cells. To verify that this EtBr stained 1 Kb band was the expected PCR product from a homologous recombination event of the targeting vector with the endogenous fps/fes gene, Southern blotting was performed using the 3' 450 bp EcoRI fragment of mfps/fes as a probe. To prepare the DNA for Southern blotting, the agarose gel was soaked in denaturing solution (1.5M NaCl, 0.5M NaOH (Fisher)) for 30 min., rinsed with dH2O, then soaked in neutralizing solution (1.5M NaCl, 0.5M Tris HCl pH 8.0) for 45 minutes. After rinsing the gel with dH2O, the DNA was transferred to Gene Screen membrane (Dupont NEN) using the PosiBlot Pressure Blotter (Stratagene) with 10X SSC for 1 hr (according to manufacturers instructions). The transferred DNA was then cross-linked to the membrane with a Stratagene UV Stratalinker set on auto-crosslink (DNA side up). ECL detection of DNA was performed using a random primer fluorescein dUTP labeling kit as directed by the manufacturer (Dupont NEN). The blot was probed with random primed fluorescein labeled DNA (450 bp EcoRI fragent) and then the probe was detected using anti-fluorescein-HRP conjugated antibodies. The PCR analysis was then performed on the individual ES cell clones for which a positive was seen in the PCR analysis of the pools. The one difference in this analysis was that once the ES cells were in the 1 ml media, a 100μl aliquot was taken to do the PCR, but the remaining 900μl was then transferred to a 6 well tissue culture plate.
Genomic Southern blotting

DNA was extracted from the PCR positive ES cell clones with 400μl of DNA extraction buffer (50mM Tris-HCl pH 8.0, 100mM EDTA, 100mM NaCl, 1% SDS) containing 1mg/ml Proteinase K and this was incubated at 55°C O/N in Eppendorf tubes. The next day, 20μl of heat inactivated 10mg/ml RNase A (Sigma) was added to the tubes and incubated at 37°C for 1-2 hrs. Using cut off P1000 tips to pipette the genomic DNA, 600μl of 1 M TrisHCl pH 8.0 saturated phenol (BDH)/chloroform (Fisher)/isoamyl alcohol (Sigma) (25:24:1) was added, rocked at rt for at least 20 min., centrifuged at 14000 rpm for 5 min. and the aqueous phase was then transferred to a new tube. This phenol/chloroform/isoamyl alcohol extraction was repeated three or four times. Then 600μl chloroform/isoamyl alcohol (24:1) was added, rocked at rt for at least 20 min., centrifuged at 14000 rpm for 5 min., and the aqueous phase was transferred to a tube containing 1 ml ethanol. The tube was inverted until a DNA precipitate formed. The precipitate was collected by spooling, washed with 70% ethanol, and air dried. The genomic DNA was resuspended in 200μl TE (10mM TrisHCl pH 8.0, 1mM EDTA) and stored at 4°C. The concentration was calculated by measuring the optical density (OD) at 260 nm in a Spectrophotometer (LKB Biochrom).

To determine which ES clones contained the K to R mutation, approximately 20μg purified genomic ES cell DNA was digested with BglII for Southern blotting analysis. Initially, the genomic ES cell DNA was diluted into 1X NEB restriction endonuclease buffer#3 for a 100μl digest and incubated at 4°C O/N. The next day, 70 units of the BglII enzyme (NEB) was added, and incubated at 37°C O/N to ensure complete digestion of the genomic DNA. The DNA was precipitated by adding 100μl TE, 200μl 5M NH4OAc (BDH), and 1 ml EtOH, mixing, and centrifuging 20 min. at rt. The pellet was washed
with 70% EtOH and air dried. The DNA was then resuspended in 30μl ddH2O and heated at 65°C before loading onto a 0.7% agarose gel, using a wide well comb to obtain compact bands. The DNA was electrophoresed at 55V until it was into the gel then at 25V O/N. To prepare the DNA for transfer to Zeta probe membrane (Biorad), the agarose gel was soaked in 0.2M HCl for 10 min., washed well with dH2O, and then soaked in denaturing solution for 45 min. After rinsing the gel with dH2O, it was soaked in neutralizing solution for 45 min. Capillary transfer to Zeta probe membrane using 10X SSC was then set up to go overnight. The DNA was cross-linked to the membrane using a Stratagene UV Stratalinker set on auto-crosslink. The membrane was then soaked in 10 mls prehybridization solution (50% Formamide (BDH), 0.25M NaH2PO4 (BDH), 0.25M NaCl, 7% w/v SDS, 1mM EDTA) at 42°C for at least 1 hr. A radioactive probe was synthesized overnight at rt in a 25μl reaction containing 100 ng of fps/fes cDNA from the vector pBLTN-4 (10) as a template (denatured 100°C for 5min. and put directly on ice for 2 min.), 5μl OLB buffer (50mM TrisHCl pH8, 5mM MgCl2, 20μM dTTP, 20μM dGTP, 20μM dCTP, 10mM 2-mercaptoethanol, 0.2M Hepes pH6.6, 5.4 OD units/ml pd(N6)), 1μl 10mg/ml BSA (Sigma), 5μl α32P-dATP (10μCi/μl or 3000 Ci/mmoll; Dupont NEN) and 1μl Klenow (NEB). The probe was spun purified using a G50 Sephadex column (Pharmacia), heat denatured for 5 min. at 100°C, and added to 10 mls fresh prehyb which was then incubated with the membrane at 42°C O/N (hybridization). The hybridization solution was removed, and the membrane was rinsed in 2X SSC. To remove non-specific hybridization, the membrane was washed in 2XSSC/0.1%SDS for 15 min. at rt, 0.5XSSC/0.1%SDS for 15 min. at rt, and 0.1XSSC/0.1%SDS for 5 to 15 min. at 65°C. The membrane was then exposed to Kodak X-ray film at -70°C for the desired time.
Generation of chimeric mice

Chimeric mice were generated by aggregation of clumps of ES cells with precompaction stage 8-cell embryos (see figure 8)(69). CD1 mice, obtained from Charles River Laboratories, were used as a source of supporting embryos in aggregation experiments, as pseudopregnant recipient females and for breeding with chimeric animals. On day 1 of the aggregation schedule, 22 day old CD1 female mice were injected intraperitoneally with 5 IU pregnant mare serum gonadotropin (PMSG) (Sigma) in the late afternoon. Two days later (day 3), these mice were set up with fertile males (‘studs’). On day 4, seminal copulation plugs were checked on the now 25 day old females in the morning, and in the afternoon, 8-10 wk old CD1 females were set up with vasectomized CD1 males. On day 5, plugs were checked for these pseudopregnant 8-10 wk old females. On day 6 (aggregation day), 2.5 day embryos were flushed from dissected oviducts of the superovulated bred young females, using M16 media (94.7mM NaCl, 4.8mM KCl (BDH), 1.2mM KH₂PO₄ (BDH), 1.2mM MgSO₄·7H₂O (BDH), 25mM NaHCO₃ (BDH), 1.7mM CaCl₂·2H₂O (BDH), 327μM sodium pyruvate (BDH), 4mg/ml BSA, 3.675mg/ml 70% DL sodium lactate (Sigma), 5.5mM D-glucose (BDH), 0.08mg/ml penicillin (GibcoBRL), 0.05mg/ml streptomycin (Sigma)). The zona pellucidae were removed from the 8-cell stage embryos using acid Tyrode’s solution (137mM NaCl, 2.7mM KCl, 1.6mM CaCl₂·2H₂O, 0.5mM MgCl₂·6H₂O (BDH), 5.5mM D-glucose, 4mg/ml polyvinylpyrrolidone (Sigma), solution pH to 2.5 with HCL), and then they were washed in M16 media. Indentations were made in the bottom of 35mm tissue culture dishes using a darning needle within M16 drops overlaid with mineral oil. These indentations allow the ES cells to be brought in close association (aggregated) with the 8 cell-stage precompaction CD1 embryos. The ES cells were prepared for
Figure 8: Illustration of the procedure used to generate transgenic mice.

The embryonic stem (ES) cell line RI was derived from cells of the inner cell mass of a blastocyst, which was generated from mating strains of mice with agouti coat colour alleles. The targeting vector was introduced into these ES cells in culture. Once an ES cell line containing the lysine to arginine mutation in \( f_{ps/fe} \) was established, clumps of these ES cells were aggregated with morula from CD1 mice. Blastula stage embryos were transferred into pseudopregnant recipient females, and sixteen days later chimeras were born. To determine if the ES cells have contributed to the germ cells of the chimera, the chimera was mated with a CD1 mouse. If germline transmission was achieved, offspring were born which had the agouti coat colour. This procedure is reviewed in "Gene Targeting - A Practical Approach" by A. L. Joyner (68).
MATE

agouti mice

BLASTOCYST

inner cell mass
trophoblastic cells

CULTURE ES CELLS

AGGREGATION

ES cells

morula from CD1 mice

CHIMERA produced

MATE

+ 

germline transmission

OFFSPRING

agouti is dominant

F1 generation
aggregation by trypsinization, and preplating for 30 min. at 37°C; the non-adherent or undifferentiated cells are used in the aggregations. Clumps of 6-10 ES cells were placed in the indentations, followed by a 8 cell-stage precompaction embryo. These aggregations were then incubated at 37°C and 5% CO₂ overnight. On day 7, compacted morulae or blastula stage embryos were transferred to the uterine horns of the pseudopregnant 8-10 wk recipient females, which had been anesthetized with 500 μl of 2.5% avertin injected intraperitoneally. Sixteen days later these recipient females gave birth to some chimeras. To determine if the ES cells had contributed to the germ cells of the chimera, the chimeras were bred to CD1 mice. If germline contribution was achieved, a litter was born with eye-pigmentation and agouti coat colour markers from ESRI cell genotype and this was the F1 generation.

Genotyping of mice

DNA was isolated from tails of each mouse. Approximately 1cm of the tail was cut from each mouse, and ear punching to number the mice was performed at the same time. The tail was incubated in 700μl of DNA extraction buffer containing 1mg/ml Proteinase K at 55°C O/N, and the DNA was subsequently purified as described in the purification of ES DNA for genomic Southern blotting. To genotype the mice, the genomic BgIII Southern blotting (described above) and the PCR strategy described for screening the ES cell clones were initially used for the F1 generation; but to distinguish between heterozygotes and homozygotes in the F2 generation, a BgIII PCR strategy was designed. This BgIII PCR strategy involves generating PCR products analogous to the region of the fps/fgs gene in which the lysine to arginine mutation was made, purifying these PCR products and then digesting them with BgIII (NEB). PCR was performed in a 60μl
reaction containing approx. 100ng template tail DNA, 39.5μl ddH2O, 10μl 10X Taq PCR buffer (Gibco BRL), 5μl 50mM MgCl₂ (Gibco BRL), 0.2μl 100mM dATP, 0.2μl 100mM dCTP, 0.2μl 100mM dGTP, 0.2μl 100mM dTTP, 2μl of the upstream primer oligo KtoR5' (5'-GACAAGTGGGTTCTGAAGCACGAGG-3')(18 pmol/μl), 2μl of the downstream primer oligo KtoR3'(5'-GACCCCGATGAGACGCACAATGTTGG-3')(16 pmol/μl), and 0.5μl Taq polymerase (Gibco BRL). PCR conditions were as follows: a ‘hot start’ at 95°C, 30 cycles (95°C for 1 min., 65°C for 1 min. and 74°C for 3 min.), 74°C for 10 min., and 4°C hold. The PCR products were resolved on a 1% regular agarose gel and a band of approx. 1 Kb could be seen by EtBr staining under UV light. The PCR products were purified using a QIAquick spin PCR purification column (QIAGEN); the products were eluted from the column in 50μl ddH₂O. The BgIII digest was set up using 17μl of this 50μl elute, 2μl NEB buffer #3 and 2μl BglIII, and incubated at 37°C for 2 hrs to ensure complete digestion. Uncut and BglIII cut PCR products were resolved on a 1% agarose gel.

**Histological examination of mouse tissues**

The mouse tissues were dissected from wild type, heterozygous, and homozygous mice at 7 weeks of age and immediately put into cold 4% paraformaldehyde (BDH)/PBS (made up according to Current Protocols in Molecular Biology). The tissues were fixed for approx. 18 hrs at 4°C, and then washed in PBS. The tissues were dehydrated and embedded in paraffin in the following manner: 70% alcohol 2 hrs, 80% alcohol 3 hrs, 95% alcohol 20 min., 98% alcohol 20 min., 100% ethanol 50 min. at 40°C, 100% ethanol 50 min. at 40°C, 100% ethanol 60 min. at 40°C, 50/50 ethanol/toluene 50 min. at 40°C, toluene 80 min. at 40°C, toluene 50 min. at 40°C, paraffin 30 min. at 40°C, paraffin 30 min. at 40°C, paraffin 30 min. at 40°C, and embedded. The
tissue/paraffin blocks were sectioned at two different levels (8 microns thick), mounted on slides, and stained with hematoxylin and eosin. The gross morphology of each tissue was examined for differences between tissues from wild type, heterozygous, and homozygous mice.

**Bone marrow flushes**

To flush the bone marrow, the dissected femur was cut at the hip bone, a 21.5 gauge needle was inserted at the knee, and cold DMEM in a 3cc syringe was pushed through the center of the bone.

The bone marrow cell count was done as follows: 100µl of the flush was added to 0.9 ml DMEM, mixed, 4µl of this was added to 4µl trypan blue (ICN) and put on the hemocytometer. To obtain the number of cells/ml, the number of cells counted in the centre grid were multiplied by the dilution factors and by $10^4$. For example, 77 cells in centre grid $\times 10 \times 2 \times 10^4 = 1.5 \times 10^7$ cells/ml. Approx. $1.5 \times 10^6$ cells were plated for macrophage cultures, approx. 20 million cells were used in the immune complex kinase reactions, and approx. $2 \times 10^5$ cells were plated in the 35mm methylcellulose culture plates.

**Methylcellulose culture**

Colony assays of hematopoietic cells were performed using methylcellulose media, obtained from Stem Cell Technologies. Complete methylcellulose media, which contains all of the required growth factors needed to give rise to cells of all the hematopoietic lineages, was initially used to assay whether or not the mutation in fps/les caused a block in hematopoiesis. The incomplete methylcellulose media with the addition of
individual growth factors (eg. IL3 (1ng), GM-CSF (3ng), M-CSF (15ng), IL6 (8ng), Epo (1U), and SCF (100ng))(R&D Systems) was used to determine if a particular cytokine signalling pathway was affected by the mutation in fps/fes. Approximately 2 X 10^5 bone marrow cells were plated in the 35mm methylcellulose culture plates. After 10 days in culture, erythroid colonies (CFU-E & BFU-E) were scored/counted; and then after 18 days in culture, the granulopoietic colonies (CFU-GM) were scored/counted, according to the Stem Cell Technologies Atlas (70). In addition, to look qualitatively at the granulopoietic colonies, some of these were plucked out of the methylcellulose, cytopun and stained with the Diff-Quik Stain Set (Baxter).
CHAPTER 3: RESULTS

Generation and Characterization of polyclonal antisera

Since the anti-Fps Q381-E566 serum (previously generated by P. Greer) was found to be cross-reactive to Fps/Fes and Fer proteins in both Western blotting and immunoprecipitation experiments, a number of polyclonal anti-sera raised against various regions of the Fps/Fes or Fer proteins were generated, as described in the Materials and Methods.

Cos-1 cell transfection analysis

To examine the specificity of the different antisera generated, a Cos-1 cell transfection analysis was performed. Cos-1 cells were lipofected with PECE expression constructs encoding the human Fps/Fes (PEF4) or murine Fer (PECE-Fer). Western blot analysis, using whole cell lysates from (1) untransfected Cos-1 cells (control), (2) fps/fes transfected Cos-1 cells, and (3) fer transfected Cos-1 cells, was used to analyze the specificity of the antisera (see figure 9). The anti-Fps Q381-E566 serum detected both P92 Fps/Fes and P94 Fer (see figure 9, panel A). The anti-Fer L97-A382 serum also detected both Fps/Fes and Fer (see figure 9, panel B). The anti-Fps V306-Q378 affinity purified (AP) serum detected Fps/Fes specifically and anti-Fer L305-A378 AP serum detected Fer specifically (see figure 9, panels C and D).

To determine if the polyclonal antisera were useful in immunoprecipitation experiments, Cos-1 cells were transfected with PECE expression constructs encoding either the human Fps/Fes (PEF4) or murine Fer (PECE Fer) protein. The Fps/Fes
Figure 9: Specificity of the polyclonal antisera generated against the various regions of the Fps/Fes and Fer proteins.

Cos-1 cells were transfected with PECE expression constructs encoding either the human Fps/Fes (PEF4) or murine Fer (PECE-Fer) protein. These cells were lysed and the proteins were resolved by SDS-PAGE. Western blotting was used to analyze the specificity of the different polyclonal antisera generated. Control (untransfected), Fps or Fer transfected Cos-1 cells are indicated above the lanes. Anti-Fps Q381-E566 detects both P92 Fps/Fes and P94 Fer (Panel A). Anti-Fer L97-A382 detects both P92 Fps/Fes and P94 Fer (Panel B). Anti-Fps V306-Q378 AP detects P92 Fps/Fes specifically (Panel C). Anti-Fer L305-A378 AP detects P94 Fer specifically (Panel D).
<table>
<thead>
<tr>
<th>Control</th>
<th>Fps</th>
<th>Fer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Fps Q381-E566</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Fer L97-A382</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Fps V306-Q378 AP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Fer L305-A378 AP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
and/or Fer proteins were then immunoprecipitated from lysates of the transfected Cos-1 cells using the different polyclonal antisera. The anti-Fps V306-Q378 serum and the anti-Fer L305-A378 were unable to immunoprecipitate Fps/Fes or Fer proteins (data not shown). Figure 10 shows the results of the immunoprecipitation experiments using preimmune sera, anti-Fps Q381-E566 serum, or anti-Fer L97-A382 serum. The blots were probed with anti-Fps V306-Q378, and then stripped and reprobed with anti-Fer L305-A378. The anti-Fps Q381-E566 serum is cross-reactive as it immunoprecipitated both Fps/Fes and Fer proteins, whereas the anti-Fer L97-A382 serum selectively immunoprecipitated Fer. The anti-Fps L102-Q378 was then generated in hopes that it selectively immunoprecipitated Fps/Fes. Initial immunoprecipitation experiments as described above have shown that this antiserum immunoprecipitated Fps/Fes and not Fer, but additional boosts are needed to increase the titre of the antiserum (data not shown).

In summary, the anti-Fps Q381-E566 serum is cross-reactive as it detected and immunoprecipitated both Fps/Fes and Fer proteins. The anti-Fer L97-A382 serum detected both Fps/Fes and Fer proteins, but this serum selectively immunoprecipitated Fer. The anti-Fps L102-Q378 detected both Fps/Fes and Fer proteins, but it selectively immunoprecipitated Fps/Fes. The anti-Fps V306-Q378 serum detected Fps/Fes specifically, and the anti-Fer L305-A378 serum detected Fer specifically (for a summary see Table 1).
Figure 10: Immunoprecipitations of Fps/Fes or Fer proteins from *fps/fes* or *fer* transfected Cos-1 cells.

Cos-1 cells were transfected with PECE expression constructs encoding either the human Fps/Fes (PEF4) or murine Fer (PECE-Fer) protein. These cells were lysed and the proteins were immunoprecipitated using preimmune sera (PI), anti-Fps Q381-E566 or anti-Fer L97-A382 sera. These immune complexes were then resolved by SDS-PAGE and Western blotted using anti-Fps V306-Q378 (Panel A) or anti-Fer L305-A378 (Panel B). The anti-Fps Q381-E566 serum immunoprecipitated both Fps/Fes and Fer proteins, whereas the anti-Fer L97-A382 serum selectively immunoprecipitated Fer.
anti-Fps L305-A378
anti-Fps Q381-E566
Pi Fps Q381-E566
anti-Fps Q381-E566
Pi Fer L97-A378
anti-Fer L97-A378

anti-Fps L305-A378
anti-Fps Q381-E566
Pi Fps Q381-E566
anti-Fps Q381-E566
Pi Fer L97-A378
anti-Fer L97-A378
Table 1: Summary of the reactivity of the different antisera generated.
<table>
<thead>
<tr>
<th>Antisera</th>
<th>Immunoprecipitation</th>
<th>Blotting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fer</td>
<td></td>
</tr>
<tr>
<td>α Fps Q381-E566</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>α Fer L97-A382</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>α Fps L102-Q378</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>α Fps V306-Q378</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>α Fer L305-A378</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
</tbody>
</table>
Mouse tissue western blots

The antisera described above have been used to look at Fps/Fes and Fer protein expression in a number of mouse tissues (see figure 11, panel B). The relative total protein loaded from each tissue was determined by staining the protein gels with Coomassie blue (see figure 11, panel A). The Western blot analysis of mouse whole tissue lysates using anti-Fps V306-Q378 AP suggested that Fps/Fes is most highly expressed in the bone marrow, which is consistent with the data in the literature. Fps/Fes was clearly expressed in the spleen, lung, uterus, liver, kidney, thymus and large intestine. Low level expression of Fps/Fes may be found in the other tissues, such as muscle, heart, brain, small intestine, stomach, testes, prostate and ovary. The mouse tissue blots probed with anti-Fer L305-A378 AP suggested that Fer is expressed ubiquitously (to some degree in all tissues analyzed). Mouse tissue blots probed with anti-Fps Q381-E566 and anti-Fer L97-A382 were also useful in analysing expression of Fps/Fes and Fer, as doublets were seen in some of the tissues examined. For example, in the anti-Fps Q381-E566 blot, P94 Fer and P92 Fps/Fes bands were seen as a doublet in the lung, liver, uterus, kidney and thymus, among others, as indicated by the double arrow. The expression of both Fps/Fes and Fer proteins in a number of mouse tissues is summarized in table 2. There may be low level expression of both Fps/Fes and Fer in some tissues that is beyond detection by Western blotting. The low level of expression of Fps/Fes in many tissues may be due to the fact that Fps/Fes is expressed in endothelial cells and thus blood vessels supplying these tissues. It was useful to determine these protein expression profiles because they indicate specific tissues where a defect may be found in transgenic mice expressing the inactive Fps/Fes kinase.
Figure 11: Fps/Fes and Fer protein expression in various mouse tissues.

After the mouse tissues were dissected and homogenized in lysis buffer, 2X SDS sample buffer was added. The proteins were then resolved by SDS-PAGE. In panel A, the coomassie blue stained gels showed the total protein loaded from each tissue (as indicated). Western detection using anti-Fps Q381-E566, anti-Fer L97-A378, anti-Fps V306-Q378 AP and anti-Fer L305-A378 AP, shown in panel B, allowed levels of Fps/Fes and Fer proteins to be assessed. Highest levels of Fps/Fes were seen in the bone marrow. Fps/Fes was also clearly expressed in the spleen, lung, uterus, liver, kidney, thymus and large intestine. Low level expression of Fps/Fes was observed in the other tissues. Fer was found to be ubiquitously expressed.
Table 2: Summary of the expression of Fps/Fes and Fer proteins in a number of mouse tissues.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fps/Fes</th>
<th>Fer</th>
</tr>
</thead>
<tbody>
<tr>
<td>lung</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>heart</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>brain</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>spleen</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>liver</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>bone marrow</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>stomach</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>small intestine</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>large intestine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>salivary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ovary</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>uterus</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>testes</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>prostate</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>muscle</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>kidney</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>thymus</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Generation of the "loss of function" mutation

Ralph Zirngibl was responsible for cloning the murine genomic fps/fes locus and restriction mapping of that locus. Ralph provided plasmids, such as pfesgen11A, pmfes1NKC11A, and pPNT450NHS. These were used in the PCR mutagenesis, and in the generation of the expression constructs, PXNK4 and PXNR24, and the targeting vector, 9KbRKS-pPNT450NHS#17, as described in the Materials and Methods.

To generate the "loss of function" mutation, a conserved lysine residue (K) in the ATP binding domain of Fps/Fes was chosen to be converted to an arginine residue (R). This lysine was chosen because it is highly conserved between protein kinases in general, and it has been shown to be critical for catalytic activity of many protein kinases (2). This lysine appears to be involved in the phosphotransfer reaction, whereby the gamma phosphate from ATP is transferred to tyrosine, serine or threonine residues on substrates in the cell (71,72). In addition, the lysine to arginine amino acid substitution has been shown to abolish the catalytic activity of a number of tyrosine kinases, such as cellular Lck (73), cellular Src (71) and the viral form of Fps/Fes (74). PCR mutagenesis was performed using mutagenic oligos designed to encode the arginine residue as described in the Materials and Methods. To generate this mutation, a single nucleotide base needed to be changed, that is an adenine (A) nucleotide needed to be converted to a guanine (G) nucleotide. This mutation also created a novel BglII restriction site. Not only has this novel BglII restriction site been useful in analyzing expression and targeting constructs for the mutation, but it has also been useful in analyzing targeted embryonic stem cell clones, and transgenic mice expressing the mutant fps/fes allele.
In vitro characterization of the mutation

To characterize the lysine to arginine mutation in vitro, murine genomic expression constructs encoding either the wild type or mutant Fps/Fes kinase were generated. As initial attempts in obtaining high levels of expression using the murine genomic constructs failed, wild type and mutant human fps/fes cDNAs expression constructs (previously generated in the lab) were used to characterize the lysine to arginine mutation in vitro. These expression constructs (PEF4 and pHFCKtoR2) were transfected into Cos-1 cells by lipofection so that the catalytic activity of the proteins could be analyzed by immune complex kinase assay. Fps/Fes proteins were immunoprecipitated using anti-Fps Q381-E566 from lysates of Cos-1 cells transfected with either wild type or mutant fps/fes cDNA expression constructs. Half of the immunoprecipitate was used to quantitate the protein by Western blotting and the other half was used to measure the catalytic activity by immune complex kinase assay. Using the Fps V306-Q378 specific antisera as a probe, Western blot analysis showed the relatively equivalent amounts of Fps/Fes protein in the immunoprecipitations (see figure 12A). Following incubation of the immunoprecipitates with gamma $^{32}$P-ATP, the catalytic activity was measured by the protein's ability to autophosphorylate. Compared to the mutant Fps/Fes protein, the wild type protein was highly tyrosine phosphorylated with $^{32}$P (see figure 12B), indicating that the wild type protein is catalytically active and the mutant is not catalytically active. In addition, the wild type Fps/Fes protein has also been shown to phosphorylate an exogenous substrate, enolase, in vitro; whereas the mutant Fps/Fes protein was unable to phosphorylate enolase (data not shown). All of these experiments suggested that the lysine to arginine mutation has essentially abolished the catalytic activity of Fps/Fes.
Figure 12: *In vitro* characterization of the lysine to arginine mutation.

The Fps/Fes proteins were immunoprecipitated from lysates of Cos-1 cells transfected with either wild type *fps/fes* (PEF4) or mutant *fps/fes* (pPHFCKR2) cDNA expression constructs. The immune complexes were resolved by SDS-PAGE and Western blotted using the anti-Fps V306-Q378 sera as a probe, shown in panel A. Relatively equal amounts of P92 Fps/Fes were observed in the immunoprecipitates of wild type or mutant Fps/Fes. The immunoprecipitates were also incubated with gamma-32P-ATP and the catalytic activity was measured by the protein's ability to autophosphorylate, shown in panel B. A radioactive Fps/Fes band was observed for the wild type protein, indicating that the wild type protein is catalytically active. This was not observed for the mutant protein, indicating that the arginine substitution has abolished the catalytic activity of Fps/Fes.
In vivo characterization of the mutation

Targeting Strategy

A targeting vector was designed to introduce the lysine to arginine mutation into the endogenous \( fps/fes \) gene in embryonic stem (ES) cells (reviewed in (75)). The targeting vector consisted of a 9 Kb long arm of homology, a neomycin resistance cassette, a 450 bp short region of homology in the 3' end of \( fps/fes \), and a herpes simplex thymidine kinase cassette (see figure 13). The lysine to arginine mutation was encoded within the 9 Kb long arm of homology in exon 14. The neomycin resistance cassette, under the control of the phosphoglycerate kinase (PGK) promoter, was needed for positive selection, and the herpes simplex thymidine kinase (tk) cassette, under the control of the phosphoglycerate kinase (PGK) promoter, was needed for negative selection. The neomycin resistance cassette was inserted in the 3' noncoding region of \( fps/fes \) to aid the PCR screen of targeted ES cell clones and to not disrupt the Fps/Fes protein produced. Therefore, compared to the wild type Fps/Fes protein, the only difference in the protein produced in the transgenic mice was the lysine to arginine mutation in the ATP binding region of the protein.

This targeting strategy involved a homologous recombination event occurring as a replacement between the vector and the endogenous \( fps/fes \) gene (for reviews see (75) and (76)). ES cell clones were generated by electroporating ES cells with the K to R targeting vector and then selecting with G418 and Gancyclovir (GANC), as described in the Materials and Methods. Both positive, G418, and negative, GANC, selection was used to enrich for homologous recombinant clones (68,77). Clones that integrated the targeting vector homologously expressed the neomycin resistance gene and were
Figure 13: Targeting Strategy.

The targeting vector designed to introduce the lysine to arginine mutation into the endogenous \textit{fps/fes} gene in embryonic stem cells, the endogenous \textit{fps/fes} locus, and the targeted locus are illustrated. This strategy involved a homologous recombination event occurring as a replacement between the vector and the endogenous \textit{fps/fes} gene, also called a double-crossover event (shown by the dotted lines). The PCR strategy used to identify G418 and GANC resistant ES cell clones that have integrated the targeting vector homologously is shown. A sense primer corresponding to the neo cassette and an antisense primer corresponding to genomic sequences not included in the targeting vector are indicated by the arrows. To determine which of the targeted ES cell clones contain the K to R mutation, a \textit{BglII} genomic Southern blot analysis was performed. \textit{BglII} sites that are significant for the genomic Southern Blot analysis using full length \textit{fps/fes} cDNA as the probe, as well as the distance between the \textit{BglII} sites are illustrated.
resistant to G418 selection, as the neomycin resistant gene encoded for a phosphotransferase that inactivated the G418. In addition, homologous recombinant clones were resistant to gancyclovir selection, as the tk gene was not expressed. In general, random integration clones were killed off during GANC selection because the herpes simplex thymidine kinase was expressed and phosphorylated gancyclovir. Since gancyclovir is structurally related to the nucleotide guanine, phosphorylated gancyclovir became incorporated into the DNA during replication, causing chain termination and cell death. Thus this double selection strategy was used to enrich for homologous recombinant clones.

**PCR Screening of ES cell clones**

To screen the G418 and GANC resistant ES cell clones for homologous recombination, PCR was performed on DNA isolated from these ES cell clones, as described in the Materials and Methods. These resistant ES cell clones were subjected to PCR analysis using a sense primer corresponding to the neo cassette and an antisense primer corresponding to genomic sequences 3' of the short arm of homology (see figure 13). It was estimated that a PCR product of approximately 1 Kb should be generated from clones in which the targeting vector had integrated homologously. The PCR products were resolved on a 1% agarose gel and Southern blotted. Hybridization was performed using the 450 bp EcoRI fragment, the short arm of the targeting vector, as a probe. The PCR analysis was used to screen 647 G418 and GANC resistant ES cell clones and amplification of the 1 Kb product was seen in 14 of those clones (see figure 14), suggesting a targeting frequency of 2%.
Figure 14: Southern Blot Analysis of the PCR screen on ES cell clones.

G418 and GANC resistant ES cell clones #47 and #48 were subjected to PCR analysis using a sense primer corresponding to the neo cassette and an antisense primer corresponding to genomic sequences 3' of the short arm of homology (see Figure 13). The PCR products were resolved on a 1% agarose gel and Southern blotted using the short arm of homology as a probe. A plasmid containing the complete genomic locus of the mutant fps/les gene (PXNR24) was used as a positive hybridization control. The 1 Kb hybridizing band in ES cell clone #48 corresponded to the expected size of the PCR product in a homologously targeted ES cell clone.
Southern blot analysis of ES cell clones

To determine which of the targeted ES cell clones contained the lysine to arginine mutation, a BgIII Southern blot using DNA isolated from these clones was performed, as described in the Materials and Methods. This blot was probed with the full length fps/fes cDNA to determine which clones contained the mutation and also to verify that the locus was intact. This analysis indicated that four of fourteen targeted ES cell clones contained the K to R mutation with no other rearrangements (see figure 15). ES cell clones with the K to R mutation in Fps/Fes showed hybridization to a 6.6 Kb BgIII fragment from the wild type fps/fes allele and a 4.6 Kb and a 2.0 Kb BgIII fragments from the mutant (K to R) fps/fes allele. ES cell clones 28, 22, 83, 103, 107, 111, among others integrated the targeting vector homologously but didn't incorporate the K to R mutation; in these clones crossover occurred downstream of the mutation. The hybridization to BgIII fragments of 1.8 Kb, 2.2 Kb, and 2.4 Kb in the fps/fes locus suggested that the locus was intact and no other rearrangements have occurred, except in clones 10 and 48 where there were additional fragments hybridizing with the full length fps/fes cDNA probe. This analysis indicated that ES19, ES60, ES39 and ES33 targeted ES cell clones contained the K to R mutation with no other rearrangements (see figure 15). PXNR24, the complete murine genomic fps/fes sequence including the K to R mutation cloned into a PECE SV40 expression plasmid, was used as a positive control for the Southern and for clones with the mutation. In the positive control lane, the 2.2 Kb BgIII fragment became a 1.8 Kb BgIII fragment due to a BgIII site in the polylinker of the PECE vector and the increased intensity of the 1.8 kb band was seen in this lane. In summary, the BgIII genomic Southern blot analysis indicated that four of the fourteen targeted ES cell clones contained the K to R mutation with no other rearrangements.
Figure 15: BglII genomic Southern Blot analysis.

Genomic DNA was extracted from the PCR positive ES cell clones and digested with BglII. The BglII restriction fragments were resolved on a 0.7% agarose gel and then Southern blotted using the full length fps/fes cDNA as a probe. The novel BglII site indicative of the K to R mutation causes a 6.6 Kb fragment to be broken down into 4.6 Kb and 2.0 Kb fragments. ESRI, untransfected ES cells, was used as a positive control. Only the 6.6 Kb band was seen in this lane as these cells have two wild type fps/fes alleles. PXNR24 was used as a control for clones containing the mutation, and the 4.6 Kb and 2.0 Kb bands were seen in this lane. ES19, ES33, ES39 and ES60 were found to be heterozygous for the mutation and no other rearrangements occurred in the fps/fes locus. These cell lines were then used in generating aggregation chimeras.
**Generation of chimeric mice**

Dr. Peter Greer was responsible for performing the aggregation experiments, including flushing of embryos, removal of the zona pellucidae, aggregation of the ES cells with these embryos, and transfer of the blastula stage embryos to pseudopregnant mice, as described in the Materials and Methods, so that the chimeric mice could be obtained quickly.

Chimeric mice were generated using three of the targeted ES cell clones: ES19, ES60 and ES39 (see Table 3 and figure 16a). One chimeric male was found to have 100% of his germ cells derived from the ES cells, not derived from the supporting CD1 embryo, as all of his offspring have the eye-pigmentation and agouti coat colour markers from ESRI cell genotype (see figure 16b). This F1 generation was genotyped using the PCR strategy described for screening for targeted ES cell clones and the BglII genomic Southern blot analysis.

**F1 and F2 generations of mice**

Once the F1 generation was genotyped, a heterozygous male was set up with a heterozygous female to obtain the F2 generation (see figure 17a). The F2 generation was genotyped at 3 weeks of age as described in the Materials and Methods. A sample of the data obtained using the BglII PCR strategy is shown in figure 18. Uncut and BglII cut PCR products were resolved on a 1% agarose gel. For a wild type mouse, only a 1000 bp band was seen, following BglII digestion. For a heterozygous mouse, three bands were seen following BglII digestion, the 1000 bp band from the wild type allele and the 700 bp and 300 bp bands from the mutant allele. For a homozygous mouse, the 700 bp and 300 bp bands were observed following BglII digestion, indicating that both fps/les...
Table 3:  Chimeras generated from three of the ES cell lines with the targeted K to R mutation in fps/fes.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Born</th>
<th># chimeras</th>
<th>wean (3 wks)</th>
<th>breed (6 wks)</th>
<th>Litters/Pups born</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES19 15/7/96</td>
<td>1/8/96</td>
<td>2</td>
<td>23/8/96</td>
<td>Sept12 22/9/96</td>
<td>X chimeric</td>
</tr>
<tr>
<td></td>
<td>10 pups</td>
<td>(1♂, 1♀)</td>
<td></td>
<td></td>
<td>X CD1</td>
</tr>
<tr>
<td>ES19 17/7/96</td>
<td>3/8/96</td>
<td>7</td>
<td>25/8/96</td>
<td>Sept 14 22/9/96</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>17 pups</td>
<td>(all ♀)</td>
<td></td>
<td></td>
<td>X 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/11 9/10/96, 0/16 2/10/96, litter in utero-nongenetic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 ♀ 12 18/10/96, ♀115 22/10/96 100% germine chimera</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X 6 0/13 7/10/96, 0/10 9/10/96, 0/15 2/11/96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X 7 0/22 9/10/96, 0/14 28/10/96</td>
</tr>
<tr>
<td>ES 60 26/7/96</td>
<td>12/8/96</td>
<td>5</td>
<td>3/9/96</td>
<td>25/9/96</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>19 pups</td>
<td>(4 ♂, 1 ♀)</td>
<td></td>
<td></td>
<td>X 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/13 10/10/96, 0/14 22/10/96, 0/12 30/10/96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/10 13/10/96, 15/10/96, 0/17 21/10/96, 0/18 4/11/96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/32 12/10/96, 15/10/96, 0/12 2/11/96, 0/17 4/11/96</td>
</tr>
<tr>
<td>ES 60 29/7/96</td>
<td>15/8/96</td>
<td>2 (1 runt)</td>
<td>6/9/96</td>
<td>26/9/96</td>
<td>X 1 low degree chimera</td>
</tr>
<tr>
<td></td>
<td>13 pups</td>
<td>(both ♂)</td>
<td></td>
<td></td>
<td>X 2 low degree chimera</td>
</tr>
<tr>
<td>ES 60 31/7/96</td>
<td>17/8/96</td>
<td>4 (1 highly)</td>
<td>8/9/96</td>
<td>28/9/96</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>15 pups</td>
<td>(2 ♂, 2 ♀)</td>
<td></td>
<td></td>
<td>X 2 low degree chimera</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1 very weak ♀)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES 39 30/8/96</td>
<td>17/9/96</td>
<td>2 (1 highly)</td>
<td>9/10/96</td>
<td>28/10/96</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>7 pups</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 16: Transgenic mice which have been generated.

Targeted ES cell lines containing the mutation were used in aggregation experiments to generate chimeras, as described in the Materials and Methods. Panel A shows four chimeras which were generated. These chimeras had different degrees of ES cell contribution, as indicated by the agouti coat colour. To test germline contribution, the chimeras were mated with CD1 mice. Panel B shows a litter born from a chimera whose germ cells were derived from the ES cell line #19. This was the F1 generation and some of these pups were determined to be heterozygous for the K to R mutation.
A. Chimeras (K to R)

B. F1 generation: pups from germline chimera
Figure 17: Transgenic mice which have been generated.

To generate the F2 generation, a female heterozygous for the K to R mutation was mated with a male heterozygous for the mutation. The offspring from this mating are shown in panel A. Mice homozygous for the K to R mutation were found in this litter. Adult females from this generation are shown in panel B. The dark brown mouse was determined to be wild type at the fps/ves locus. The light grey mouse was determined to be heterozygous for the K to R mutation, and the white mouse was homozygous for the mutation.
A. F2 generation (K to R): heterozygote X heterozygote

B. Females from F2 generation (+/+, +/-, -/-)
Figure 18: PCR genotyping of transgenic mice.

PCR was carried out on tail DNA using *fps/fes* primers, KtoR5' and KtoR3', spanning the novel BgIII restriction site. The purified PCR products were digested (or not) with BgIII, as described in the Materials and Methods. Uncut or BgIII digested PCR products were resolved by agarose gel electrophoresis and visualized by EtBr staining. The uncut products migrated as a 1000 bp band. If the PCR products contained the novel BgIII restriction site, indicative of the mutant allele, then this 1000 bp band was broken down into 700 bp and 300 bp fragments. Mouse number 2.18 has two wild type *fps/fes* alleles. Mouse number 2.16 is heterozygous for the lysine to arginine mutation, and mouse number 2.17 is homozygous for the mutation.
alleles contain the K to R mutation (see figure 18). This result indicated that mice homozygous for the lysine to arginine mutation were viable. No obvious abnormalities, physical or behavioral, could be observed between mice of the three genotypes (see figure 17b).

**Phenotypic analysis of transgenic mice**

Marion Arnold was responsible for the embedding and sectioning of mouse tissues, as well as the preparation and H & E staining of the histological slides. Dr. Sandy Boag, a lung pathology expert, was responsible for providing advise when differences seen in the lung sections were not reproducible. Dr. Sam Ludwin, a neuropathology expert, was responsible for the examination of the brain for obvious abnormalities.

**Histological examination**

Since the lysine to arginine mutation in Fps/Fes may alter the function of cells in tissues such as the brain, lung, kidney, thymus, liver, spleen, uterus and intestine, these tissues were initially examined for gross morphological changes. Initially, differences were observed in the lung, large intestine and uterus. As shown in figure 19, the lung appeared more cellular in +/- and -/- lung sections, compared to +/+ lung sections. However, this was not a reproducible observation, as in subsequent experiments the lung sections from +/+, +/- and -/- mice all appeared as cellular as the -/- lung sections in figure 19. In consultation with Dr. Sandy Boag, it was concluded that the artifact observed in the initial experiment was due to differences in perfusion or expansion of the lungs at the time of the dissection. Initially, differences were also observed in the large intestine where there appeared to be more goblet-type
Figure 19: Histological examination of the lung.

Lungs were dissected from +/+, +/-, and -/- K to R mice. These tissues were then fixed, embedded and sectioned. This figure is a composite of H & E stained sections of the lungs from +/+, +/-, and -/- K to R mice (as indicated). The lung sections from the mice expressing the mutant Fps/Fes protein appeared more cellular, compared to the wild type lung sections. This observation was not reproducible in subsequent experiments, as lung sections from all three genotypes appeared very cellular (like pictures shown for -/- mice in figure). This artifact was determined to be due to differences in perfusion or expansion of the lungs at the time of the dissection. Left panels are 40X magnification. Right panels are 100X magnification.
cells in the intestine from the +/+ mouse (data not shown). However, this was not a reproducible observation, as in the next experiment, dissecting the same parts of the large intestine from mice of the three genotypes revealed that the differences seen in the initial experiment were due to dissecting different parts of the large intestine from different mice. The initial examination of the brain was conducted in collaboration with Dr. Sam Ludwin. Immunohistochemistry analysis using an antibody to glial fibrillary acidic protein (anti-GFAP) suggested no difference in cell death between transverse sections of the brain from +/+ and +/- mice (data not shown). With respect to the kidney, thymus, liver and spleen, no gross morphological differences were observed in the tissue sections from +/+, +/- and -/- mice (data not shown).

Fertility of homozygous mutant mice

The fertility of a homozygous mutant male appeared to be normal, as he sired normal size litters (i.e. 12 pups and 14 pups). In contrast, the fertility of the homozygous mutant females appeared to be abnormal. A number of different breeding pairs were set up. The homozygous mutant females either gave birth to extremely small litters (i.e. 2 pups, 4 pups) or no offspring at all (i.e. 0 pups with two different females). This was the first sign that there was a female fertility defect.

To investigate whether this fertility defect was due to an abnormality in the ovary, uterus or placenta, initially the histology slides were reexamined. The ovary section from the -/- mouse suggested that the fertility defect is not due to an abnormally functioning ovary (see figure 20). The maturation of oocytes as well as the degeneration of oocytes or the formation of corpus luteum were observed. In addition, ovulation, the release of an oocyte into the oviduct, was observed in this section (see figure 20).
Figure 20: Examination of female fertility in -/- K to R mice.

Histological examination of the ovary section (H & E stained) from a -/- K to R mouse showed the maturation of oocytes as well as the formation of corpus luteum (top panel - 80X magnification). The bottom panel shows the release of an oocyte into the oviduct (200X magnification). This suggested that the ovary from the -/- K to R mouse was functional. Consequently, the female fertility defect is probably at the level of the uterus or placenta.
Consequently, the female fertility defect may be at the level of the uterus, placenta, or a combination of the two. The histology slides provided no evidence of obvious abnormalities in the non-pregnant uterus (data not shown). Initial experiments suggested the embryos or fetuses were dying after six days implantation in the homozygous mothers. To determine what abnormalities in the uterus and/or placenta are causing the fertility defect in the homozygous mutant females, embryos including the placenta and uterus should be dissected from pregnant mice of all three genotypes at a variety of gestational periods.

**Hematopoiesis/Methylcellulose assays**

To determine whether or not the mutation in fps/fes caused a block in hematopoiesis, approximately 2 X 10^6 bone marrow cells flushed from +/+, +/−, and −/− mice were grown in complete methylcellulose media. After the required number of days in culture, there was no difference in the number of erythroid colonies (CFU-E & BFU-E) or granulopoietic colonies (CFU-GM) arising from +/+, +/−, or −/− bone marrow cells. In addition, to look qualitatively at the granulopoietic colonies, some of these were plucked out of the methylcellulose, cytopun and stained with the Diff-Quik Stain Set (Baxter). Granulocytes and monocytes were observed in the cytopins of the complete methylcellulose cultures of +/+, +/− and −/− bone marrow cells (see figure 21).

To determine if a particular cytokine signalling pathway was affected by the mutation in fps/fes, colony formation in methylcellulose was examined in the presence of defined growth factors, such as IL3, GM-CSF, M-CSF, IL6, Epo, and SCF. The initial experiment suggested that there was a difference in the number of colonies growing up.
Figure 21: Evidence that hematopoiesis is 'normal' in -/- K to R mice.

Bone marrow cells were cultured in complete methylicellulose for 18 days. Granulopoietic colonies were then plucked out of the methylicellulose, cytospun and stained with Diff-Quik (Baxter). This is a picture of monocytes grown from -/- bone marrow cultures (500X magnification). Similar cells were observed in +/- and +/+ cultures.
between +/-, +/- and -/- bone marrow cultures when the individual growth factors were used. In particular, there was a relative decrease in the number of granulopoietic colonies grown from +/- and -/- cultures compared to +/+ cultures, when IL-3, GM-CSF or M-CSF was solely added. This observation suggested that in the complete methylcellulose cultures other cytokine signaling pathways may be compensating for the loss of Fps/Fes function. Whether Fps/Fes function is absolutely required in IL-3, GM-CSF or M-CSF signaling leading to erythroid and granulopoietic colony formation needs to be further investigated.

*Western blots and Immune complex kinase of bone marrow from transgenic mice*

It was important to determine whether or not the transgenic mice express the inactive Fps/Fes kinase, and this was performed using bone marrow flushed from mice of the three genotypes. After the bone marrow cells were counted, 2X SDS sample buffer was added to the lysates and the proteins were resolved by SDS-PAGE. A Western blot of these bone marrow lysates, probed with anti-Fps Q381-E566, suggested that Fps/Fes and Fer protein levels were unaltered in the transgenic mice (see figure 22, Panel A). Since the neomycin resistance cassette was inserted in the 3' region of fps/fes where a weak cis-acting element has been found, the unaltered protein levels suggested that transcription and translation of fps/fes were not greatly affected by the insertion of the neomycin resistance cassette. Following immunoprecipitation of the proteins using anti-Fps Q381-E566 or anti-Fer L97-A382, the immune complexes were incubated with gamma-\(^{32}\)-P-ATP. The anti-Fps Q381-E566 lanes in figure 22, panel B showed a decrease in Fps/Fes activity in the +/- bone marrow lysate and a further decrease in Fps/Fes activity in the -/- bone marrow lysate (see figure 22, Panel B). Therefore, in
Figure 22: Evidence that the transgenic mice express the inactivated kinase.

Bone marrow cells were flushed from femurs of mice of the three genotypes. After counting the cells, they were lysed in KLB. Some of the clarified lysate was used to quantitate the amount of Fps/Fes and Fer proteins in the lysate by Western blotting. The rest of the lysate, approx. 20 million cells, was used in immune complex kinase assays. Fps/Fes or Fer proteins were immunoprecipitated from the bone marrow lysates, using anti-Fps Q381-E566 or anti-Fer L97-A382, and then incubated with gamma-^{32}P-ATP. In panel A, Western blotting using anti-Fps Q381-E566 showed relatively equivalent amounts of Fps/Fes and Fer proteins from bone marrow cells flushed from mice of each genotype. In panel B, the anti-Fps Q381-E566 immunoprecipitates incubated with gamma-^{32}P-ATP showed a decrease in Fps/Fes activity in the +/- lane and a further decrease in Fps/Fes activity in the +/- lane, compared to the +/- lane. This result suggested that in the transgenic mice activity of the kinase was at the relative levels expected.
A.

- control
- +/- bone marrow
- +/- bone marrow
- +/- bone marrow

\[ \alpha_{Fps} \text{ Q381-E566} \]

B.

- control \( \alpha_{Fps} \text{ Q381-E566} \)
- +/- \( \alpha_{Fer} \text{ L97-A382} \)
- +/- \( \alpha_{Fps} \text{ Q381-E566} \)
- +/- \( \alpha_{Fer} \text{ L97-A382} \)
- +/- \( \alpha_{Fps} \text{ Q381-E566} \)
- +/- \( \alpha_{Fer} \text{ L97-A382} \)

\[ \text{P94 Fer} \quad \text{P92 Fps} \]
the transgenic mice, activity of the kinase was at the relative levels expected. It was interesting to notice that in the -/- immune complex kinase reactions Fer activity may be increased compared to +/- and +/- immune complex kinase reactions (see figure 22, Panel B), although this was not consistently observed. In addition, the P92 Fps/Fes band in the -/- anti-Fps Q381-E566 kinase reaction lane was more intense than expected, considering results from the in vitro characterization of the mutation. This suggested that Fer may be tyrosine phosphorylating Fps/Fes in trans (see figure 22, Panel B). Whether or not Fer activity is increased and whether or not Fer is tyrosine phosphorylating Fps/Fes in the -/- mice need further investigation.
CHAPTER 4: DISCUSSION

Implications of generating and characterizing the anti-Fps and anti-Fer antisera

Since the anti-Fps Q381-E566 serum was found to be cross-reactive to both Fps/Fes and Fer proteins in Western blotting and immunoprecipitation experiments, it was very important to generate and characterize antisera that were specific for Fps/Fes or Fer. The anti-Fps V306-Q378 serum was found to detect Fps/Fes specifically, and the anti-Fer L305-A378 serum was found to detect Fer specifically. The anti-Fer L97-A382 serum selectively immunoprecipitated Fer. The anti-Fps L102-Q378 selectively immunoprecipitated Fps/Fes, but additional boosts are needed to increase the titre of this last antiserum. These antisera have been used in immunohistochemistry and immunofluorescence analyses (14), as well as in the analysis of the expression and activity of Fps/Fes and Fer in normal and transgenic mice. In addition, these antisera will be useful in the dissection of signal transduction pathways involving Fps/Fes or Fer, in particular in the determination of whether a specific protein is associating with Fps/Fes or Fer. In summary, generating antisera that distinguishes between Fps/Fes and Fer proteins was a useful and successful endeavor.

Generation of transgenic mice

Initially it was important to demonstrate that the lysine to arginine mutation abolished the catalytic activity of Fps/Fes in vitro as it then justified the generation of the transgenic mice with this inactivating mutation.

Gene targeting has been used to introduce specific mutations into embryonic stem
cells for a number of years (for reviews see (75,76,78,79). A number of factors have been found to affect targeting frequency in homologous recombination experiments. For example, targeting efficiency is dependent on the location of the target in the genome and the use of isogenic DNA, that is DNA from the same strain of mouse as that of the ES cells. In addition, the total length of homology in the targeting vector relative to the target locus greatly affects the targeting frequency. In general, a 2-fold increase in the length of homology results in a 20-fold increase in targeting frequency (76). Generally, homology regions should be greater than 1 Kb on each side of the neomycin resistance gene, with a total homology of 6 Kb or greater; but it is beneficial to have a greater length of homology on the long arm. Researchers have found a dramatic (200-fold) increase in targeting frequency with an increase in homology from 1.3 to 6.8 Kb (80), and a dramatic decrease in frequency below 0.25 Kb (81). Hasty et al. also found that a total homology of less than 1.7 Kb was insufficient to generate targeted events. Although 472 bp of homology on the short arm was found to be as efficient as 1.2 Kb for a double-crossover or gene replacement event in the formation and resolution of crossover junctions (80).

In the generation of the targeting vector to introduce the “loss of function” mutation into the endogenous fps/les gene, the total length of homology was 9.45 Kb, which exceeded the 6 Kb total homology recommended. In addition, the greater length of homology was on the long arm. The short arm of homology was 450 bp, but that length of homology was sufficient to give a double-crossover event. This gene replacement strategy gave a targeting frequency of 2%, which was a good targeting frequency and within the range expected. When using similar targeting strategies, researchers have found targeting frequencies of 2.5% generally (59,82), but ranging from 1.2% to 10%
This result further supports the use of a targeting strategy in which at least 6 Kb of homology to the target locus and double selection are used. The BglII genomic Southern blot analysis showed which of the targeted ES cell clones contained the mutation. In addition, the BglII genomic Southern blot ensured that the locus was intact and no other rearrangements occurred in the ES cell clones used in the generation of the transgenic mice.

Finally, with respect to generating germline chimeras, the quality of the ES cell line is one of the key factors. A "good" ES cell line is indicated when the degree of chimerism obtained exceeds 50% and the contribution to tissues is widespread (83). Excessive passaging of the ES cells, that is 20 passages or approx. 100 cell generations, will allow subsets of aneuploid cells (like trisomies) to form (83). The maintenance of a cell line with a normal, diploid genotype is crucial in generating germline chimeras (83). If high degree chimeric animals are not being obtained using a particular ES cell line, then it is possible that the cell line has an abnormal genotype and karyotype analysis should be performed to rule out aneuploidy. The ES cell line #19 in general contributed highly to a variety of tissues in the chimeric animals generated; therefore, it was not surprising that one of these chimeras had 100% of his germ cells derived from the ES cell line #19. In summary, generating a "good" ES cell line was key in obtaining the germline chimera and consequently mice heterozygous for the K to R mutation.
**Phenotype and Future perspectives**

**Role of Fps/Fes in blood vessel formation**

There was no overt phenotype in the mice heterozygous or homozygous for the K to R mutation. Since previous evidence suggested that Fps/Fes plays a role in vascular endothelial cell proliferation, the K to R mutation in Fps/Fes was thought to possibly cause a decrease in vasculogenesis and/or angiogenesis. Since mice homozygous for the K to R mutation were found to be viable, Fps/Fes activity appears to not be essential for blood vessel formation during embryogenesis. Endothelial cells appear to be sensitive to activated tyrosine kinases (84), and the phenotype seen in the ‘MF’ transgenic mice may reflect this sensitivity. However, Fps/Fes may be playing a role in blood vessel formation in pregnancy, since there is a female fertility defect that has not been completely characterized. It is possible that Fps/Fes is required in endothelial cells in response to stress. Experiments to investigate the response of endothelial cells to vascular injury could be conducted to address this possibility. Fps/Fes may also play a role in the secretion of a number of different factors from endothelial cells. Endothelial cell lines established from mice of the three genotypes could be useful in addressing this question. Following stimulation of these cells, secretion of a factor such as von Willibrand factor could be measured; a significant difference in the amount of the factor secreted would suggest the catalytically inactivated Fps/Fes protein may be altering the secretion function of these cells. The function of Fps/Fes in endothelial cells is obviously not clearly understood, but analysis of these transgenic mice have provided many avenues for further investigation.
Role of Fps/Fes in hematopoiesis

Initial methylcellulose culturing evidence suggests that Fps/Fes activity is not essential for hematopoiesis, the generation of mature granulocytes and monocytes, thus the mutation in $fps/fes$ did not cause a block in hematopoiesis. Peripheral blood analysis showed no obvious differences in white blood cell counts, platelet counts or red blood cell counts. Since a leukemic phenotype may not be revealed in mice of 6 weeks of age, peripheral blood and bone marrow analyses should also be performed using mice of 1 and 2 years of age. Collaborations with Dr. R. Auerbach's laboratory in which numbers of myeloid progenitors and mature myeloid cells will be examined with the use of cell surface markers, will substantiate the theory that Fps/Fes activity is not essential for hematopoiesis. Since Fps/Fes activity may not be required for the differentiation of myeloid cells as mature granulocytes, macrophages and mast cells exist in the transgenic mice, then whether the mutation in $fps/fes$ alters the function of these cells should be determined. For example, the catalytically inactive Fps/Fes protein may affect the ability of the basophils and/or mast cells to secrete chemical mediators of inflammation. Neutrophils or eosinophils may not be attracted to sites of acute inflammation or cells may not be able to phagocytose bacteria or antigen-antibody complexes. Finally, the K to R Fps/Fes may affect the phagocytosis or secretion function of macrophages. In addition, since Cas has been identified as a substrate of Fps/Fes (43) and Jucker et al. suggest Fps/Fes may play a role in cell adhesion and cell-cell interactions during immune responses of macrophages, then whether or not the mutation in Fps/Fes affects the communication between hematopoietic cells should also be investigated. In summary, the function of Fps/Fes in hematopoietic cells is not clearly understood, but these transgenic mice provide the tools needed to address this question.
Role of Fps/Fes in secretion

The mutation in Fps/Fes may affect secretion in a variety of tissues and cell types, such as vascular endothelial cells and hematopoietic cells as previously discussed. In addition, how the mutation affects secretion in the brain and uterus should also be addressed. In the brain, Fps/Fes may be involved in the secretion of neurotransmitters as immunofluorescence analysis on the 20Y neuroblastoma cell line showed that Fps/Fes localizes to a perinuclear region, coincident with the Golgi apparatus, on the side of the cell that was extending neurite outgrowths, along the extended neurite, and at the end-terminal region of the neurite outgrowth (14). Consequently, it will be interesting to examine whether the K to R Fps/Fes transgenic mice have a brain defect or neuromuscular abnormality. With respect to the uterus, since fps/fes has been found to be expressed in glandular epithelial cells of the uterus, whether or not secretion by these cells is affected by the loss of Fps/Fes activity should be determined.

Role of Fps/Fes in pregnancy

The female fertility defect suggests Fps/Fes activity is involved in pregnancy. Mutations in a number of genes have been found to be associated with female infertility in both humans and mice. These “Maternal Effect” mutants can affect female fertility in a number of ways. For example, a mutation in the cystic fibrosis gene has been associated with female infertility resulting from the production of thick cervical mucus which inhibits sperm motility (85). In addition, transgenic female mice homozygous for a “null” mutation in the prolactin receptor gene were found to be infertile due to the failure of the embryos to implant (86). Transgenic mice homozygous for a mutation in the zona pellucida gene, Zp3, were infertile due to the lack of zona pellucida (87).
The lysine to arginine mutation in \textit{fps/fes} appears to be a "Maternal Effect" mutant, but it doesn't appear to affect the oocyte or the implantation of the embryos. The infertility in these transgenic mice may be due to abnormalities in secretion from the glandular epithelial cells of the uterus or failure to establish a functional placenta. Extensive vascularization is needed in the formation of a functional placenta such that it is able to support the growth of the embryo/fetus; consequently, since Fps/Fes function in endothelial cells is not clearly understood, further analysis of these transgenic mice may demonstrate a requirement of Fps/Fes activity in the establishment of a functional blood supply needed to support pregnancy.

To investigate when and why the embryos/fetuses are dying in the +/- K to R mothers, histological analysis of the pregnant uterus at different stages of pregnancy should be conducted. Immunohistochemistry, using the anti-Fps V306-Q378 antisera, on uterus/placental sections from +/-, +/-, and +/- K to R mice will help investigate whether there is a certain cell type missing in the +/- pregnant uterus. For example, uterine macrophages or glandular epithelial cells may be absent in the +/- pregnant uterus. A pregnancy defect was found in the osteopetrotic (op/op) mouse, demonstrating the requirement for M-CSF (or CSF-1) in female fertility and no macrophages were apparent beyond day 14 of pregnancy (88). Fps/Fes may act downstream of the M-CSF receptor and this analysis may elucidate a signaling pathway that is essential in pregnancy. Whether or not these uterine macrophages are functioning normally should also be investigated. In summary, further investigation into the cause of this female infertility in these transgenic mice will provide great insight into the biological function of \textit{fps/fes}.  

81
Cytokine signaling pathways

To investigate whether or not the catalytically inactive Fps/Fes protein is causing an alteration in cytokine signaling, cytokine stimulation experiments, using bone marrow cells flushed from +/+, +/-, and -/- K to R mice, should be extensively conducted. In particular IL-3, GM-CSF and M-CSF stimulations on primary macrophages should be performed since initial methylcellulose colony assays suggested a relative decrease in the number of granulopoietic colonies arising from +/- and -/- cultures compared to +/+ cultures, when the individual cytokine was added. These experiments may also help to elucidate signaling pathways in which Fps/Fes is involved. For example, following cytokine stimulation of a primary macrophage culture, one can determine what proteins are phosphorylated in wild type cells but have a decrease in phosphorylation in cells expressing the K to R allele. This could give you an idea of the substrates of Fps/Fes in a variety of cell types and the resulting effect of altering that signaling pathway.

Functional redundancy with Fer

It was important to discover that the transgenic mice expressed the inactive Fps/Fes kinase, as this validates all of the results obtained with respect to generating the transgenic mice. However, the immune complex kinase assays on bone marrow cells flushed from mice of the three genotypes raised a number of questions with respect to Fer activity. For example, is Fer activity increased and is Fer tyrosine phosphorylating Fps/Fes in the -/- mice? This needs further investigation, but it suggests that Fer may be compensating for the loss of Fps/Fes activity in the transgenic mice. Is functional redundancy occurring? To address that question, the Fps/Fes K to R transgenic mouse
needs to be crossed with the Fer 'knockout' mouse, currently being generated in the lab. This double knockout may reveal phenotypes that were masked in the Fps/Fes K to R single knockout due to functional redundancy with Fer.

In conclusion, this thesis illustrates the generation and characterization of transgenic mice expressing a targeted inactivating mutation in the $fps/fes$ gene. This research has given us new insight into the biological function of this proto-oncogene. Investigating the phenotype of mice carrying the K to R mutation in $fps/fes$ is expanding our knowledge of how Fps/Fes is involved in hematopoiesis, blood vessel formation, secretion and pregnancy. These transgenic mice provide the tools to allow the elucidation of signaling pathways in which Fps/Fes is involved. This research has revealed many questions that need to be addressed with respect to the function of Fps/Fes as well as with respect to the involvement of Fer in Fps/Fes signal transduction pathways. Investigation into the cause of the female infertility defect is of great importance to pursue, as this may demonstrate the requirement of Fps/Fes activity in a particular aspect of blood vessel formation, hematopoiesis, or secretion. This research may also lead to the association of female infertility problems in humans to other mutations in $fps/fes$. This research was very successful and hopefully the transgenic mice will eventually be useful in finding treatments for female infertility problems or for a disease like leukemia.
References


