Generation and Characterization of MAPKAPK5-Deficient Mice

Dissertation

von der Fachbereich Chemie

der Universität Hannover

zur Erlangung des Grades einer

DOKTORS DER NATURWISSENSCHAFTEN

Dr. rer. nat.

genehmigte Dissertation

von MSc. Yu Shi

geboren am 01. Mai 1963, in Heilongjiang, V.R. China

2002

Referent:	Prof. Dr. H. Meyer
Korreferent:	Prof. Dr. M. Gaestel, Prof. Dr. E. Ungewickell
Tag der Promotion:	29. Oktober 2002

Abstract

The p38 mitogen-activated protein kinase (MAPK) pathway, like the c-Jun N-terminal kinase (JNK) MAPK pathway, is activated in response to cellular stress and inflammation and is involved in many fundamental biological processes. MAPKAP kinase 5 (MK5) is one of several kinases that are regulated through direct phosphorylation by p38 MAPK. To study the role of the p38 MAPK pathway, and the function of MK5 *in vivo*, we have generated mice with a germline mutation of the MK5 gene. The mice have been characterised by Southern blot analysis, RT-PCR, sequencing, as well as Western blot analysis and protein kinase assays, which all demonstrated that the targeted disruption of the MK5 gene resulted in a null allele.

MK5-deficient mice were viable and fertile. The intrinsic MK5 kinase activity is absent in MK5-deficient mice and could be detected in wt mice, but we were not able to activate this kinase with typical p38 pathway stimuli in our experiments. After immunization, the gene targeted mice form abnormal large germinal centres. Most cytokines are normal in the MK5-deficient mice. In resting cells GFP-MK5 is located in nucleus. Treatment with arsenite results in nuclear export of MK5 after 90 min in the Hela cells only in the case of co-transfection with p38. Participation of MK5 in MAP kinase signalling pathways and its functional significance have been discussed.

Keywords: MAPKAP-Kinase 5/ p38-MAPK/ deficient mice

Zusammenfassung

Die p38 Mitogen-aktivierte- Proteinkinase (MAPK)- Kaskade, wie z.B. die c-Jun-N-terminale- Kinase(JNK)-MAPK-Kaskade, wird als Antwort auf zellulären Stress und Entzündungen aktiviert und spielt eine große Rolle in grundlegenden biologischen Prozessen. MAPKAP-Kinase 5 (MK5) ist eine von verschiedenen Kinasen, welche durch eine direkte Phosphorylierung durch die p38-MAPK reguliert wird. Um die Rolle der p38-MAPK-Kaskade und die Funktion der MK5 in vivo zu untersuchen, konstruierten wir Mäuse mit einer Keimbahnmutation des MK5-Gens. Die Mäuse wurden durch Southern-Blot-Analyse, RT-PCR, Sequenzierung, Western-Blot-Analyse und Proteinkinase-Assays charakterisiert, welche alle zeigten, dass die gezielte Zerstörung des MK5-Gens zu einem Null-Allel führte.

Die MK5-defizienten Mäuse waren lebensfähig und fruchtbar. Die intrinsische MK5-Kinaseaktivität fehlte bei den MK5-defizienten Tieren und konnte bei den WT-Mäusen nachgewiesen werden, allerdings konnten wir keine weitere MK5-Aktivierung mit den typischen Stimuli der p38-Kinasekaskade erzielen. Nach der Immunisierung zeigten die genveränderten Mäuse abnormal vergrößerte Keimzentren. In unstimulierten Zellen ist GFP-MK5 im Kern lokalisiert. Die Stimulierung mit Arsenit führt nur im Fall einer Cotransfektion mit p38 zu einem Kernexport der MK5 nach 90 Minuten in Hela Zellen. Die Beteiligung der MK5 in MAP-Kinase-Signalkaskaden und ihre funktionelle Bedeutung werden diskutiert.

Schlüsselwörter: MAPKAP-Kinase 5/ p38-MAPK/ defizienten Mäuse

Acknowledgements

I express my heartfelt thanks to Prof. Dr. Matthias Gaestel and Dr. Alexey Kotlyaov, who gave me the warm-hearted helps in designing and guiding the whole project.

And heartfelt thanks to all of my colleagues, who worked or still work in Gaestel's group. I thank Dr. Armin Neininger for scientific advice and helpful discussions; Frau Beate Schöne for taking care of the mice and isolation of the DNA; Frau Kathrin Laaß for technical support and transportation of the mice; Frau Dorothee Krone and Frau Stefanie Feldhege for technical support; Prof. Hans-Dieter Volk and Dr. Rolf Eckert (Berlin-Charite) for the measurement of cytokines; Prof. Ugo Moens and Dr. Ole Morten Seternes (University of Tromsø, Norway) for some expression vectors and offering me doing some kinase assays in their laboratory.

Special thanks to my wife and all of my family members who tried their best to support me.

Hannover, May 2002

Yu Shi

Index

Page

Abstract (in English) 2
Zusammenfassung (Abstract in German) 3
Acknowledgments 4
Index 5
List of Tables and Figures 8
Abbreviations 10
Thesis: Generation and Characterization of MAPKAPK5-Deficient Mice 13
I. Introduction: p38 MAP Kinase Cascade: Pathway, Regulation and Their Functions 13
1. MAPKs 13
1.1 MAPKs 13
1.2 General Functions 13
1.3 Gene Targeting in p38 MAPKs Cascade 15
2. p38 MAPKs 17
3. Regulation of the p38 signalling pathway 18
3.1 Extracellular Stimuli 19
3.1.1 Environmental Stresses and Pathogens 19
3.1.2 Cytokines 19
3.1.3 Growth factors 20
3.1.4 TCR/CD3 complex and the CD28 costimulatory receptor, CD40 and Fas/CD95 22
3.1.5 Autophosphorylation and Autoactivation of p38α 23
3.2 The Inhibitor SB203580 23
3.3 p38 MAPKs Upstream Activators: MKKs 24
3.4 Further Upstream Activators 26
3.5 p38 MAP Kinases Inactivation 27
4. p38 MAPKs Targets: Downstream substrates of p38 MAP kinases 30
4.1 Protein Kinases 30
(1) MK2 and MK3/3pk 31
(2) MK5/PRAK 32
(3) MNK1 33
(4) MSK1 33
4.2 Transcription Factors 33
(1) ATF2 34
(2) MEF 2C and 2A 34
(3) TCF Proteins: Elk1 and SAP1 35
(4) C/EBP Family: CHOP and C/EBPβ 35
4.3 Others 35
(1) cPLA2 35
(2) Stathmin/Op18 35
5. Subcellular Localization of p38 MAPKs and Their Downstream Targets 35
6. p38 Pathway Functions 36
6.1 Cytokines: Production and Gene Expression 36
(1) IL-1β and TNF-α 36
(2) IL-4 36

(3) IL-5 36
(4) IL-6 37
(5) IL-8 37
(6) IL-12 37
6.2 Other Gene Expressions 37
(1) IE Genes 37
(2) VCAM-1 37
(3) iNOS 38
(4) COX-2 38
6.3 Apoptosis 39
6.4 Cell Growth and Differentiation 39
II. Materials and Methods 41
1. Materials 41
Cell lines and mouse strains 41
Regents for cell culture 41
Plasmids 42
Oligos and their sequences 42
Probes for hybridisation 43
Antibodies, enzymes and chemicals 43
Kits 44
Equipments 44
2. Methods 45
2.1 Construction of targeting vectors 45
2.1.1 Isolation of MK 5 genomic DNA 45
2.1.2 Cloning of the 6.5 kb Bam HI and the 9 kb Sac I fragments of MK5 gene 45
2.1.3 Construction of the targeting vectors 46
2.1.4 DNA analysis 49
2.2 Generation of MK5 knockout mice 49
2.2.1 Culture of ES cells 49
2.2.2 Preparation of the linearized targeting vector 50
2.2.3 Electroporation and Selection of the ES cells 50
2.2.4 Picking the clones 51
2.2.5 Freezing ES clones 51
2.2.6 Screening of the clones 52
2.2.7 Preparation of positive clones for microinjection 52
2.2.8 Generation of MK5 knockout mice 53
2.2.9 Establishment of MK5 deficient embryonic fibroblast cell line (MK5-/-MEF) 53
2.3 Genotype of the mice 53
2.3.1 Isolation of genomic DNA from mouse tails 53
2.3.2 Genotype mice by PCR 54
2.3.3 Genotype mice by Southern Blot 54
2.4 Characterization of the mouse 54
2.4.1 Isolation of total RNA from tissues or from cells 54
2.4.2 Western immunoblotting analysis 55
2.4.3 MK5 Kinase Assay 55
2.4.4 Induction of endotoxic shock 56

Page

2.4.5 Immunization 57
2.4.6 Measurement of cytokines production 57
2.4.7 Statistical analysis 57

III. Results and Discussions
1. Analysis of MK 5 Gene and Construction of Targeting Vector 58
2. Generation and Characterisation of MK5-Deficient Mice 64
(1) Screening and Genotyping of the Mice by PCR 66
(2) Characterisation of the Gene Targeting Mice by Southern Blot Analysis 66
(3) Characterisation of the Gene Targeting Mice by Northern Blot Analysis, RT-PCR and Sequencing 67
(4) Characterisation of the Gene Targeting Mice by Western Blot Analysis, Protein Kinase Assay and subcellular localization 70
3. Analysis of MK5-Deficient Mice 76
4. Further Analysis of MK5-Deficient Mice 82
IV. References 83
V. Declaration 93
VI Curriculum Vitae 95

List of Tables and Figures

Page

Part I: Introduction

Table 1-1: Phenotypes of MAPKK and MAPK ko mice (Chang et al., 2001) 14
Table 1-2: Properties of p38 group MAP kinase members (Ono et al., 2000) 18
Table 1-3: Environmental Stresses and Some Pathogens of the p38 Activation 19
Table 1-4: The p38 activity of representative inhibitors 24
Table 1-5: The sensitivity profile among protein kinases to SB203580 25
Table 1-6: Names of MKKs 26
Table 1-7: MKKKs of p38 pathway 27
Table 1-8: MKKKs of p38 pathway and their other names 27
Table 1-9: Members of MKPs 28
Fig. 1-1: The tentative classification of MKPs (Tanoue et al., 2001b) 29
Table 1-10: Some substrates of MK2/3 and their phosphorylation residues 31
Table 1-11: MK5 and PRAK 32
Table 1-12: Some cell lines mentioned in the introduction 40

Part II: Materials and Methods

Table 2-1: Cell lines and mous strains 41
Table 2-2: Regents for cell culture 41
Table 2-3: Plasmids 42
Table 2-4: Oligonucleotides and their sequences 42
Table 2-5: Probes for hybridisation 43
Table 2-6: Antibodies, enzymes and chemicals 43
Table 2-7: Kits 44
Table 2-8: Equipments 44

Part III: Results and Discussions

Fig. 3-1: Phylogenetic tree of six MAPKAPKs 58
Fig: 3-2: Analysis of MK5 genomic DNA and ES cell DNA by PCR 59
Table 3-1: Location of primer 59
Table 3-2: Results of PCR analysis of MK5 genomic DNA and ES cell DNA 59
Fig. 3-3: Structure of MK5 gene and strategy applied to mutate the MK5 gene 61
Fig. 3-4: Relevant sequence of the MK5 gene 62
Fig. 3-5: Structure of the 15.5 kb MK5 genomic DNA 63
Fig. 3-6: Southern blot analysis of mouse genomic DNA with probe P2 63
Fig. 3-7: Southern blot analyses of the electroporated ES cell clones 64
Table 3-3: 20 chimeric mice 64
Table 3-4: Birth rate of the mice 65
Fig. 3-8: Screening and genotyping mice by PCR 66
Fig. 3-9: Southern blot analysis of the mice with probe P4 67
Fig. 3-10: Northern blot analysis of macrophages with probe P5 67
Fig. 3-11: RT-PCR of macrophages 68
Fig. 3-12: MK5 ko mice coding domain sequence 68

Fig. 3-13: Alignment of MK5 protein sequences of wt and ko 69
Fig. 3-14: Western blot analysis 70
Fig. 3-15: MK5 kinase assay in MEFs 71
Fig. 3-16: MK2 kinase assay in MEFs 72
Fig. 3-17: MK5 kinase assay in transfected MEFs 72
Fig. 3-18: Export of MK5 74
Fig. 3-19: Export of MK2 75
Fig. 3-20: Effect of LPS/D-galatosamine on the survival in MK5 mutant mice 76
Fig. 3-21: Cytokines production by spleen cells 77
Table 3-4: Production of IL-4 and IL-12 of spleen cells derived from wt (+/+) and MK5 ko (-/-) mice 78
Fig. 3-22: Leukocyte antigens 78
Fig. 3-23: Germinal centre formation in spleens of wt and MK5 ko mice after immunization for 12 days 80
Fig. 3-24: Production of immunoglobulins after immunization 81

Abbreviation

Ab	antibody
Ag	antigen
ATF	activating transcriptional factor
AP–1	activating protein 1
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BPAEC	bovine pulmonary artery endothelial cells
BMK 1	big MAP kinase1
CD	cluster of differentiation
CDD	common docking domain
C/EBP	CCAAT/enhancer-binding protein
CHOP	cAMP response element-binding protein-homologous protein
ConA	concanavalin A
COX-2	cyclooxygenase-2
cPLA2	cytosolic phospholipase A 2
CRE	cyclic-AMP response element
CREB	cyclic-AMP response element-binding protein
CSAID	cytokine-suppressant anti-inflammatory drug
CSF-1	colony stimulating factor-1
CT6	cytokine-dependent T cell line
DLK	dual leucine zipper-bearing kinase
EAT	mouse Ehrlich ascites tumor cells
eIF-4E	eukaryotic initiation factor 4E
Epo	erythropoietin
ERKs	extracellular signal-related kinases
ES	embryonic stem
FGF	fibroblast growth factor
FMLP	fMet-Leu-Phe
FLSs	fibroblast-like synoviocytes
FRET	fluorescence resonance energy transfer
GADD153	growth arrest DNA damage 153
GC	germinal center
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HIV-1	HIV type 1
HOG	high osmolarity glycerol response
HUVEC	human umbilical vein endothelial cells
HSP	heat shock protein
IE gene	immediate-early gene

IGF-I	insulin-like growth factor I
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
JNK	Jun amino-terminal kinases
ko	knockout
LAMPf	*Mycoplasma fermentas* lipid-associated membrane proteins
LN	lymph node
LMB	Leptomycin B
LPS	lipopolysaccharide
LSP 1	Lymphocyte-specific protein 1
MALP-2	Mycoplasma fermentans membrane-associated lipopeptide macrophage-activating lipopeptide-2
MAPK	mitogen-activated protein kinase
MAPKAPK(or MK)	MAP kinase-activated protein kinase
MEF	mouse embryonic fibroblast
MEF	myocyte enhancer factor
MEK	mitogen-activated protein kinase kinase
MEKK	mitogen-activated protein kinase kinase kinase
MKK	MAPK kinase
MKKK	MKK kinase
MKPs	MAP kinase phosphatases
MLK 2	the protein kinases mixed-lineage kinase-2
MNK1	MAPK interaction protein kinase 1
MSK1	mitogen- and stress-activated protein kinases-1
NES	nuclear export signal
NLS	nuclear localization signal
NGF	nerve growth factor
NO	nitric oxide
Op18	oncoprotein 18
PAF	platelet-activating factor
PC 12	rat pheochromocytoma cell line
PCD	programmed cell death
PDGF	platelet-derived growth factor
PHAS-1	phosphorylated heat- and acid-stable protein-1
PMA	phorbol myristate acetate
PMNs	polymorphonuclear neutrophils
PP2C	a serine/threonine protein phosphatase type 2C
PRAK	p38-regulated/activated kinase
PTPase	protein tyrosine phosphatase
SaOS2	osteoblastic cells
SAPKs	stress-activated protein kinases
SB	Southern blot hybridisation

SEB	staphylococcal enterotoxin
SLF	Steel locus factor
SRF	serum response element
TAK1	TGFβ-activated protein kinase 1
TCF	ternary complex factor
TGF-β	transforming growth factor beta
THP-1	human monocyte
TNF-α	tumor necrosis factor-α
TNFR	tumor necrosis factor receptor
UV	ultraviolet
VCAM-1	endothelial adhesion molecule vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
wt	wild type

I. Introduction: p38 MAPK Cascade: Pathway, Regulation and Their Functions

1. MAPKs

1.1 MAPKs

Mitogen-activated protein kinases (MAPKs) are evolutionary conserved enzymes that connect cell-surface receptors to critical regulatory targets within cells. The MAPK pathways convert receptor signals into various outputs. In mammalian cells, four distinct MAPKs have been identified:

(1) extracellular signal-related kinases (ERK)-1/2
(2) c-jun N-terminal kinases or stress-activated protein kinases (JNK1/2/3, or SAPKs)
(3) p38 MAPKs (p38 α/β/γ/δ)
(4) ERK5 or big MAP kinase 1 (BMK 1)

MAPK cascades are composed of three tiers of sequentially activating protein kinases, which commonly are referred to as MAPK, MAPK kinase (MKK) and MAPKK kinase (MKKK). An activated MKKK phosphorylates and activates a specific MKK, which in turn activates a specific MAPK:

MAPKKK (MKKK or MEKK) → MAPKK (MKK or MEK) → MAPK

MAPKs are activated through phosphorylation on both threonine and tyrosine residues at the Thr-Xaa-Tyr (TXY) dual phosphorylation motif. This motif is located between the subdomains VII and VIII of the kinase catalytic domain (Hanks et al., 1995) and confers specificity to each MAPK subgroup, allowing for their independent regulation.
For ERK, JNK and p38 MAPKs, X = Glu (E), Pro (P), and Gly (G) respectively.

T***E***Y for ERKs
T***P***Y for JNKs
T***G***Y for p38 MAPKs

These dual specificity kinases are relatively specific for each MAPK subgroup, allowing for their independent regulation.

Normaly MAPKs are activated by specific MKKs (MAPKKs), but there is some cross-talk between the different cascades:

MKK1/2 (or MEK1/2) for ERK1/2
MKK4/7 (JNKK1/2) for the JNKs
MEK5 for ERK5
MKK3/6 for the p38 MAPKs

Each MKK, however, can be activated by more than one MKKK, thus increasing the complexity and diversity of MAPK signal transduction.

1.2 General Functions

Each MAPK recognizes a number of substrates allowing the regulation of many processes. Hence, once activated, MAPKs need to find the correct target. The ERK1/2 signal pathway is the main signal transduction pathway, mostly activated by growth-related stimuli (Cobb et al., 1991; Sugden et al., 1997). The JNKs are activated in response to a number of cellular stresses, such as high osmolarity and oxidation (Ip et al., 1998). The ERK5 signal pathway regulates serum-induced early gene expression (Kato et al., 1997). Finally the p38 MAPKs are involved in immune responses and inflammation, cell growth, differentiation and apoptosis. The actual roles of each MAPK cascade are highly cell type and context dependent.

In order to learn more about MAPKs functions, it is very important to investigate the normal and pathophysiological functions in the whole organism. In recent years, with the analysis of the mutant mouse strains produced by gene targeting, we have gained new understanding about the functions of MAPK. Transgenic mice have been produced with various deficiencies in MAPK signal cascades particularly at the MAPKK and MAPK level. Chang et al. have summarized the phenotypes of MAPKK and MAPK knockout (ko) mice (table 1-1). What has been learnt from MAPKK and MAPK gene targeting is summarized below.

Table 1-1: Phenotypes of MAPKK and MAPK ko mice (from (Chang et al., 2001), extended)

MKK/MAPK	Phenotypes	Similar to
MEK1	defective placental vasculaization (Giroux et al., 1999)	ERK2?
MKK4	defective liver development (Ganiatsas et al., 1998)	c-Jun knockout (Hilberg et al., 1993)
MKK7	embryonic lethality of unknown cause (Dong et al., 2000)	
MKK3	defective IL-12 production (Lu et al., 1999)	
SEK1(MKK4)	defective proliferation and IL-2 production in peripheral T Cells (Nishina et al., 1997)	
ERK1	defective T-cell development (positive selection) (Pages et al., 1999)	MEK1 dn negative transgenics
JNK1	defective T-cell differentiation to Th2 cells (Dong et al., 1998)	
JNK2	defective T-cell differentiation to Th1 cells (Yang et al., 1998)	
JNK1 or JNK2	defective T-cell proliferation and IL-2 Production (Sabapathy et al., 1999)	JNK1 dn negative transgenics MKK4 ko
JNK1 or JNK2	defective activation induced death of thymocytes (Sabapathy et al., 1999)	JNK1 dn negative transgenics
JNK1 & JNK2	IL-2 overproduction (Dong et al., 2000)	MKK7 ko
JNK1 & JNK2	neural tube disclosure (Kuan et al., 1999) (Sabapathy et al., 1999b)	
JNK3	resistance to excitotoxic neuroal cell death (Yang et al., 1997)	c-JunA63/73 knockin (Behrens et al., 1999)
p38α	placental defect (trophoblast cells) (Adams et al., 2000)	
p38α	insufficient production of erythropoietein (Tamura et al., 2000)	

dn=dominant-negative; ko=knockout

1.3 Gene Targeting in p38 MAPKs Cascade

(1) p38 MAPKs

Within the p38 MAPK subfamily, only p38α has been inactivated by gene targeting. This resulted in embryonic lethality. In an inbred C57BL6/J background, p38α deficiency resulted in lethality before day 11 of gestation, owing to defective placental development (Adams et al., 2000). When the placental defect was rescued by aggregation of tetraploid (p38α +/+) and diploid (p38α -/-) morulae, p38α -/- embryos developed to term and were normal in appearance. This indicated that the defect was secondary to insufficient oxygen and nutrient transfer across the placenta (Adams et al., 2000).

A role for p38α in various aspects of cardiogenesis including the regulation of cardiomyocyte differentiation, apoptosis, and hypertrophy has also been suggested (Clerk et al., 1998; Wang et al., 1998 ; Kolodziejczyk et al., 1999; Davidson and Morange, 2000). However, Adams et al. did not detect any obvious changes in differentiation, proliferation, and apoptosis of cardiomyocytes in the different embryonic stages analysed (Adams et al, 2000).

It has also been reported that some p38α -/- embryos die between embryonic days 11.5 and 12.5, and that those developing past this stage have normal morphology but are anaemic owing to failed definitive erythropoiesis, caused by diminished erythropoietin (Epo) gene expression (Tamura et al., 2000). It has been suggested that p38 affects Epo gene expression at the posttranscriptional level, most likely through mRNA stabilization. This indicates that the p38α plays a critical role linking developmental and stress-induced erythropoiesis through regulation of Epo expression.

The function of p38 signalling in normal development has also been examined in *Drosophila.* A *Drosophila* p38 MKK gene, licorne (*lic*), was isolated in yeast (Suzanne et al., 1999). It was found that both in yeast and in cell cultures, *lic* can activate vertebrate p38 specifically, suggesting that at least some components of the p38 pathway are conserved in *Drosophila*. In *Drosophila*, oogenesis provides a workable genetic model to study pattern formation and the establishment of asymmetry in the egg. The polarity of the oocyte and the future embryo depends on several cell communication events between the germ line and the somatic tissues, which signal the specific localization of few determinants at the anterior [bicoid (*bcd*)], posterior [oskar (*osk*)], and dorsal [gurken (*grk*)] regions of the developing oocyte (Ray and Schüpbach, 1996). It has been reported that *lic* mutations provoke polarity defects in the eggshell and embryo, as a result of reduced activity of two localized determinants, *osk* and *grk*. This fact indicates that the *Drosophila* p38 MAPK pathway is essential for oogenesis (Suzanne et al., 1999).

The p38 MAPK pathway was thought to be mostly involved in inflammation and stress, but with the analysis of p38α deficient mice and mutations in *lic* in *Drosophila*, it has come to light that p38α additionally has critical developmental functions.

(2) MKK3

MKK3 activates p38 MAPKs. MKK3 deficient mice were viable without obvious abnormalities, but showed an impaired type I cytokine immune response. MKK3 ko

macrophages were defective in the production of interleukin-12 (IL-12) and interferon-γ (IFN-γ) following immunization with protein antigens and also *in vitro* differentiation of naive T cells was greatly reduced (Lu et al., 1999). Though this defect occurs at the transcriptional level, the identity of p38 MAPKs responsive transcription factor is still not clear (Lu et al., 1999).

(3) MAPKAPK2 and MAPKAPK5 (MK2 and MK5)

Over the past few years, our laboratory (Prof. Matthias Gaestel's group) worked on the downstream targets of p38 MAPKs and successfully produced MAPKAPK2 (MK2), and MAPKAPK 5 (MK5) ko mice (subject of this work). Currently, double ko mice (MK2 and MK5) are under construction. We found that MAPKAPK ko mice are viable and fertile, grow to normal size and do not exhibit obvious behavioural defects. They do show some defects in the production of some cytokines. The production of tumor necrosis factor-α (TNF-α) and IFN-γ are markedly reduced and the release of IL-1β and IL-6 are also reduced in MK2 ko mice (Kotlyarov et al., 1999). Under certain experimental conditions, the production of IL-4 was markedly reduced in MK5 ko mice - about 75 % inhibition (details follow in this thesis). These data indicated that different populations of TH cells could be affected in MAPKAPK ko mice.

(4) Activating transcriptional factor 2 (ATF-2)

ATF-2 is a substrate of p38 MAPKs (Jiang et al., 1997; Stein et al., 1997; Cuenda et al., 1997; Goedert et al., 1997). ATF-2 mutant mice had decreased postnatal viability and growth, with a defect in endochondral ossification at epiphyseal plates. The animals had ataxic gait, hyperactivity and decreased hearing. In the brain, there were reduced numbers of cerebellar Purkinje cells, atrophic vestibular sense organs and enlarged ventricles (Reimold et al., 1996). The widespread abnormalities in ATF-2 mutant mice demonstrate its absolute requirement for skeletal and central nervous system development, and for maximal induction of select genes with CRE sites, such as E-selectin (Reimold et al., 1996). Later experiments with ATF-2 mutant mice also showed that ATF-2 is critical for the full induction of adhesion molecules (E-selectin, P-selectin, endothelial adhesion molecule and vascular cell adhesion molecule-1 -VCAM-1), cytokines (TNF-α, IL-1β and IL-6) and chemokine genes early in an immune response, but the absence of ATF-2 results in an over-exuberant response after 1–2 days (Reimold et al., 2001).

(5) cAMP-responsive element-binding protein (CREB)

Another transcriptional factor, CREB, is a substrate of mitogen- and stress-activated protein kinase 1 (MSK1), a protein kinase directly downstream-to p38 MAPKs. It has been shown that CREB α/δ-deficient mice (partial mouse knockout for CREB) are deficient in long-term memory (Bourtchuladze et al., 1994), while the complete CREB knockout leads to perinatal lethality as well as defects in T cell development (Rudolph et al., 1998).

(6) MSK1/2

A mouse embryonic stem (ES) cell line with targeted disruption of the MSK1 gene locus has been developed. It was shown that mitogen-induced phosphorylation of CREB at Ser133 was greatly reduced in these ko cells (Arthur and Cohen, 2000). MSK1, MSK2

and double knockouts (both MSK1 and MSK2) mice were produced recently (Wiggin et al., 2002). Experiments using embryonic fibroblasts derived from these ko mice showed that MSK1 and MSK2 are required for the stress-induced phosphorylation of CREB and ATF1 in primary embryonic fibroblasts. In contrast, mitogen-induced phosphorylation of CREB and ATF1 was greatly reduced but not completely abolished. The mitogen-and stress-induced phosphorylation of CREB at Ser133 has been linked to the transcription of several immediate early genes, including c-fos, junB, and egr1. The ko of both MSK1 and MSK2 resulted in a 50% reduction in c-fos and junB gene transcription in response to anisomycin or ultraviolet (UV) radiation but only a small reduction in response to tetradecanoyl phorbol acetate (TPA) or epidermal growth factor (EGF) in fibroblasts. The transcription of egr1 in response to both mitogenic and stress stimuli, as well as stress-induced apoptosis, was unaffected in the MSK1/MSK2 double knockout (Wiggin et al., 2002).

2. p38 MAPKs

p38 was first isolated as 38-kDa protein, which was rapidly tyrosine phosphorylated in response to lipopolysaccharide (LPS) stimulation. Molecular cloning of the protein revealed that it was a MAPK family member (Han et al., 1994). p38 was identified as an upstream kinase of MK2 in interleukin-1 (IL-1) or arsenite-stimulated cells (Freshney et al., 1994; Rouse et al., 1994). Other p38 isoforms were identified (p38 β/γ/δ) and it was demonstrated that they share 74%, 60% and 57% amino acid sequence identity with p38 (now named p38α) (Jiang et al., 1996; Lechner et al., 1996; Cuenda et al., 1997; Wang et al., 1997). All of the p38 MAPKs have a 12 amino acid activation loop comprising the "TGY" motif. They are activated by the phosphorylation on the Thr and Tyr residues of the TGY motif (Raingeaud et al., 1995; Doza et al., 1995). Like p38α, p38β is also inhibited by SB203580, but p38γ and p38δ are not affected by this compound (Cuenda et al., 1997).

p38α and p38β genes are widely expressed in most tissues (Jiang et al., 1996). However, p38γ and p38δ were found to be differentially expressed in the tissues analysed: p38γ was mainly expressed in skeletal muscle (Lechner et al., 1996; Li et al., 1996) and p38δ in lung, kidney, testis, pancreas and small intestine (Kumar et al., 1997).

The domains of the kinases which bind the substrates and activators of all the MAPKs are termed the common docking (CD) domain. This domain varies among the p38 MAPK isoforms. The CD domain motifs are: DPDD for p38α
DPED for p38β/γ
DPEE for p38δ

However, the CD domain alone does not determine the docking specificity. For this reason, the concept of a docking groove has been proposed. By inspection of the steric structure of p38 and ERK2, a groove comprising both the CD domain and the ED (Glu160 and Asp161 of human p38) or TT (Thr156 and Thr157 of rat ERK2) site have been identified (Tanoue et al., 2001). The docking groove of p38 and ERK MAPKs may be utilized for the docking interactions with the MAPK-interacting molecules (MKKs, MKPs and MAPKAPKs). Each amino acid in both the ED (TT) site and the CD domain may affect the docking specificity in a distinct way (Tanoue et al., 2001).

The properties of p38 MAPKs were summarized in table 1-2.

Table 1-2: Properties of p38 group MAP kinase members (Ono et al., 2000)

p38 isoforms	Other names	No. of Amino acids	Size of mRNA(kb)	Apparent MW(kDa)	Sensitivity to SB203580
p38α	p38, CSBP,SAPK2 MPK2, RK, Mxi2	360	3,5	38	+
p38β	p38-2, p38β2	364	2,5	39	+
p38γ	ERK6, SAPK3	367	2,0	43	-
p38δ	SAPK4	366	1,8	40	-

3. Regulation of the p38 signalling pathway

3.1 Extracellular Stimuli

p38 MAPKs are activated by extracellular stimuli which include environmental stresses, pathogens, cytokines, growth factors, and other factors.

3.1.1 Environmental Stresses and Pathogens

p38 MAPKs transduce the signals of environmental and some pathogenic stresses. Table 1-3 summarizes these stimuli.

Pathogens activating p38 MAPKs include macrophage-activating lipopeptide (MALP-2) and *Mycoplasma fermentas* lipid-associated membrane proteins (LAMPf). *Mycoplasma fermentans*, a human pathogen, is a potent activator of monocytes and macrophages. A synthetic analog of the Mycoplasma fermentans membrane-associated lipopeptide macrophage-activating lipopeptide-2 (sMALP-2, 200 nM) can also activate p38 and other MAPKs. This activation induces mRNA synthesis and protein secretion of IL-1β and TNF-α in human monocytes/macrophages and the murine macrophage cell line RAW 264.7. The specific p38 inhibitor SB203580 abrogated both cytokine synthesis and NF-κB and AP-1 transactivation in response to sMALP-2 (Garcia et al., 1998). LAMPf, (1 µg/ml) or LPS were also reported to induce IL-8 secretion by THP-1 cells (human monocytes/macrophages) and human polymorpho- nuclear neutrophil (PMN). Both ERK1/2 and p38 pathways are involved in IL-8 production. SB203580 efficiently blocked IL-8 production in both cell types in response to either LAMPf or LPS (Marie et al., 1999).

Table 1-3: Environmental stresses and some pathogens of the p38 activation

Environmental Stresses and Pathogens	Cell type			Reference
	Hela	293	Other cells	
Heat Shock		45 °C 15 min		Meriin et al., 1998
			CCL39 44 ^{o}C 20 min	Dorion et al., 1999
NaCl		500 mM		Wang et al., 1997b
Sorbitol	300 mM			Raingeaud et al., 1995
			cardiac myocytes 500 mM	Clerk et al., 1998
UV irradiation	40 J/m^2			Raingeaud et al., 1995
		8,6 J/ m^2		Wang et al., 1997b
H_2O_2		500 μM		Wang et al., 1997b
			Jurkat 100 μM	Lechner et al., 1996
LPS			RAW264.7 THP-1 10 ng/ml	Han et al., 1994
			Monocytes/ Macrophages 10 ng/ml	Han et al., 1994
			CHO 10 ng/ml	Raingeaud et al., 1995
Anisomycin		50 ng/ml		Wang et al., 1997b
Na_3VO_4		1 mM		Wang et al., 1997b
PMA	10 nM			Raingeaud et al., 1995
			HL-60 cells 160 nM	Schultz et al., 1997

3.1.2 Cytokines

There are some cytokines which lead to activation of p38 pathway, such as TNF-α, IL-1/2/7/17/18, TNF-like-1 (TL-1) and granulocyte-macrophage colony-stimulating factor (GMCSF).

(1) TNF-α and TL1:

The cytokine TNF-α was found to stimulate the p38 MAPK signalling cascade in human umbilical vein endothelial cells (HUVEC). TNF-α increased the activity of the p38 substrate MK 2 and the subsequent phosphorylation of the small heat shock protein 27 (Hsp27) about two to three fold (Pietersma et al., 1997). In osteoblasts, significant activation of p38 MAPK was observed following treatment with IL-1 and TNF-α and similar results were obtained using primary bovine chondrocytes and an SV40-immortalized human chondrocyte cell line (Kumar et al., 2001).

The activation pathway from TNF-α to p38 was demonstrated as follows: TNF-α binds to its type-1 receptor (TNFR1) which recruits TNFR-associated death domain protein (TRADD). TRADD can also bind two additional signal transducers: TNFR-associated factor-2 (TRAF2) and receptor interacting protein (RIP). RIP can also bind TRAF2 and, accordingly, TNF treatment is thought to result in the formation of a TRADD·RIP·TRAF2 complex . RIP or germinal centre kinase (GCK) can interact with TRAF2 and then can activate MAPKs via MKK6/p38 or MEKK1/MKK4/JNK pathways (Yuasa et al., 1998).

TL1 is a member of the TNF cytokine family and it can induce apoptosis in bovine pulmonary artery endothelial cells (BPAEC). TL1 up-regulated Fas expression in BPAEC at 8 and 24 h after treatment, and significantly activated JNK and p38 MAPK (Yue et al., 1999).

(2) Interleukins: IL-1/2/7/17/18

In KB cells, IL-1 can activate p38 MAPK (Freshney et al., 1994). In human neutrophils the MKK3/6-p38 MAPK cascade is selectively activated by IL-1β (maximal activation by 300 U/ml after 10 min), and activation of this cascade mediates IL-1β-induced superoxide release and up-regulation of CD11b and CD15 (Suzuki et al., 2001).

IL-2, and another T cell growth factor, IL-7, activate both JNK and p38 MAPKs in the murine cytokine-dependent T cell line, CT6 (Crawley et al., 1997). Stimulation of normal human articular chondrocytes with IL-17 induced nitric oxide (NO) production, concomitant with an increase in transcripts and de novo translation products of the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) genes. The IL-6 gene was also up-regulated. ERK, JNK and p38 MAPK, all respond to the activation of IL-17 in chondrocytes and the DNA binding activity of NF-κB was also significantly induced (Shalom-Barak et al., 1998). IL-18 can activate the p38 MAPK pathway in the chronically infected U1 monocytic cell line. IL-18 increased HIV type 1 (HIV-1) and IL-8 production and the induction was inhibited by a p38 specific inhibitor (Shapiro et al., 1998).

(3) granulocyte-macrophage colony-stimulating factor (GM-CSF)

TNF-α and GM-CSF activate ERK and p38 MAPK. Both ERK and p38 MAPK cascades contribute to the ability of TNF-α and GM-CSF to prime the respiratory burst response in human polymorphonuclear neutrophils (PMNs) (McLeish et al., 1998).

3.1.3 Growth Factors

The growth factors involved in the activation of p38α include: transforming growth factor β (TGFβ), colony stimulating factor-1 (CSF-1), erythropoietin (EPO), Steel locus

factor (SLF), fibroblast growth factor (FGF), insulin-like growth factor I (IGF-I), nerve growth factor (NGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF).

(1) TGF-β (transforming growth factor β)

TGFβ-activated kinase (TAK1) is a TGF-activated Ser/Thr kinase, which can activate p38 MAPK in COS7 cells (Moriguchi et al, 1996). Later, it was found that TAK1 mainly activates JNK in 293 cells. TAK1 is a physiological regulator of the JNK and p38 MAPK pathways, but not the ERK pathway. It is likely that the induction of apoptosis by TGF-β is through JNK activation (Wang et al., 1997).

(2) hemopoietic growth factors: colony stimulating factor-1 (CSF-1), Steel locus factor (SLF) and erythropoietin (Epo)

Activation of p38 MAP kinase is involved not only in responses to stresses, but also in signalling by growth factors that regulate the normal development and function of cells of the immune system. The hemopoietic growth factors, including SLF, CSF-1, and IL-3, activate p38 MAP kinase and its downstream kinase, MK2, in mast cells and hemopoietic cells (Foltz et al., 1997). This activation occurs through two distinct classes of receptors. IL-3 acts through a hemopoietin receptor, while CSF-1 and SLF act through a receptor with intrinsic tyrosine kinase activity. Although IL-4 also acts on a hemopoietin receptor, it failed to activate p38 MAPK (Foltz et al., 1997).

Another hemopoietic growth factor, Epo, and IL-3 were also reported to activate p38 MAPK and JNK in mouse hematopoietic progenitor cells, but this activation could proceed through a kinase other than MKK3/6 and MKK4 (Nagata et al., 1997). Epo and IL-3 normally regulate growth and differentiation of erythroids and hematopoietic progenitors.

(3) fibroblast growth factor (FGF)

FGF activates p38 MAPK and its substrate in SK-N-MC (fibroblast) cells. Activation of the p38 pathway by FGF regulates gene expression at a cyclic-AMP response element (CRE) by stimulating the transcriptional activity of CREB (Tan et al. 1996).

(4) insulin-like growth factor I (IGF-I)

IGF-I binding to its receptor can rescue SH-SY5Y human neuroblastoma cells from high glucose-mediated programmed cell death (PCD). The high glucose conditions can stimulate both p38 and JNK tyrosine phosphorylation and nuclear translocation. Interestingly, IGF-I activated p38 was independent of glucose treatment, while IGF-I inhibited both glucose-mediated JNK phosphorylation and nuclear translocation. The activation of p38 and deactivation of JNK by IGF-I may lead to the protection from the apoptosis (Cheng et al., 1998). IGF-I was also reported to prevent apoptosis by activating p38 MAPK in pheochromocytoma (PC12) cells (Pugazhenthi et al., 1999).

(5) nerve growth factor (NGF) and NGF withdrawal

It has been reported that NGF withdrawal leads to sustained activation of the JNK and p38 enzymes and inhibition of ERKs, which are critical for induction of apoptosis in rat

PC-12 pheochromocytoma cells (Xia et al., 1995). This indicates that the dynamic balance between growth factor-activated ERK and stress-activated JNK-p38 pathways may be important in determining whether a cell survives or undergoes apoptosis. It was also found that NGF (100 ng/ml) could induce sustained activation of p38 and inhibition of the p38 pathway blocks cell differentiation in PC12 cells, suggesting that p38 plays an essential role in neuronal differentiation in PC12 cells (Morooka and Nishida, 1998).

Some scientists reported that activation of the p38 pathway may mediate apoptosis. Both in Rat-1 fibroblasts and in differentiated PC12 cells, in the presence of NGF, p38 activity and the number of apoptotic cells was very low (approximately 1.0%). After NGF withdrawal, p38 activity was selectively elevated and apoptosis increased to 15% (Kummer et al., 1997).

(6) platelet-derived growth factor (PDGF)

Incubation of airway smooth muscle cells with PDGF induces the activation of p38 MAPK (Pyne et al., 1997).

(7) vascular endothelial growth factor (VEGF)

VEGF is a potent chemotactic agent for endothelial cells. In primary cultures of HUVEC, VEGF can stimulate both the ERK and p38 MAPK pathways. Activation of the p38 pathway results in actin re-organization and cell migration (Rousseau et al., 1997).

(8) basic fibroblast factor (bFGF) and forskolin :

It is shown that p38 MAPK and ERK pathways can be strongly activated by bFGF and, to a lesser extent by forskolin, in murine F9 cells. The activation of MAPKs will later activate cholecystokinin (CCK) gene promoter (Hansen et al., 1999).

3.1.4 TCR/CD3 complex and the CD28 costimulatory receptor, CD40 and Fas/CD95

p38 MAPK may be fully activated in mouse T cell clones by signalling via either CD3 or CD28, but CD3/CD28 costimulation does not further enhance the amount of p38 MAPK activation (DeSilva et al., 1997). In contrast, stimulation of CD28 fails to activate p38 MAPK, but synergizes with CD3 stimulation to fully activate p38 MAPK in preactivated proliferating T cells (Salmon et al., 1997). However, it has also been reported that p38α can be activated by CD28 stimulation alone in purified human $CD4^+$ peripheral blood T cells (Schafer et al., 1999).

T cell proliferation and cytokine production usually require stimulation via both the TCR/CD3 complex and the CD28 costimulatory receptor. It has been shown that p38 MAPK activation mediates both TCR- and CD28-induced signalling in primary mouse T cells. Ligation of TCR or CD28 results in only modest p38 MAPK activation, whereas TCR and CD28 synergize upon coligation to elicit enhanced p38 MAPK activation. PMA/$Ca2^+$ ionophore costimulation, which mimics TCR/CD28-mediated signalling, fully activates p38 MAPK in primary mouse T cells, but not JNK (Zhang et al., 1999).

Cross-linking CD40 rapidly stimulated the p38 MAPK-MK2 pathway in both human tonsillar B cells and multiple B cell lines. SB203580 treatment completely prevented

MK2 activation and strongly perturbed CD40-induced tonsillar B cell proliferation. SB203580 also selectively reduced CD40-induced CD54/ICAM-1 expression, whereas CD40-dependent expression of CD40 and CD95/Fas and four CD40-responsive genes cIAP2, TRAF1, TRAF4/CART and DR3 were unaffected (Craxton et al., 1998). However, CD40-mediated NF-κB binding was not affected by SB203580, suggesting that NF-κB may not be a direct target for the CD40-induced p38 MAPK pathway. This showed that the p38 MAPK pathway is required, at least in part for CD40-induced NF-κB activation and that the induction of CD40-responsive genes occurs via both p38 MAPK-dependent and -independent pathways (Craxton et al., 1998).

3.1.5 Autophosphorylation and Autoactivation of p38α

Very recently, a new activation mechanism for p38α has been reported. It has been found that interaction of p38α with TAB1 [transforming growth factorβ-activated protein kinase 1 (TAK1)-binding protein 1] leads to autophosphorylation and activation of p38α (Ge et al., 2002). Further experiments demonstrated that p38α kinase activity is not required for TAB1 binding and the effects of TAB1 on p38α are independent of its effects on TAK1. This indicated that TAB1-mediated p38α phosphorylation is most likely an intramolecular reaction.

It was shown that TAB1-induced p38 phosphorylation was sensitive to SB203580 *in vitro* and *in vivo*. Therefore, it was also examined whether TAB1-dependent p38α phosphorylation induced by extracellular stimuli was sensitive to SB203580. SB203580 inhibited p38α phosphorylation induced by both TNF or peroxynitrite in HEK 293 cells.

The effect of SB203580 on anisomycin-induced p38α phosphorylation was less pronounced, and SB23580 had almost no effect on hyperosmolarity (sorbitol)-induced phosphorylation of p38α. In RPMI 8226 cells (a human B cell line), SB203580 treatment inhibited CpG- and LPS-induced phosphorylation of p38α, but had no effect on phosphorylation induced by bacterial lipoprotein via various toll-like receptors (Ge et al., 2002). This indicated that SB203580 functioned differently in the cells treated with different stimuli. It does not really function differentially – it always inhibits p38, so it does not inhibit activation by MKK3/6 but autoactivation of p38. Thus, the differential sensitivity to SB203580 is most likely a reflection of differing p38α activation mechanisms.

This finding provides an example of signal transduction that is controlled not only by enzymes, but also by nonenzymatic adapters, scaffolds and other "inert" proteins. The autoactivation of p38 MAP kinases facilitated by interaction with regulatory molecule(s) could be an important alternative activation pathway operating in parallel with kinase cascades in regulating intracellular signalling (Ge et al., 2002).

3.2 The p38 Inhibitors

A series of pyridinyl imidazoles, which were originally identified by their ability to suppress inflammatory cytokine synthesis, can specifically inhibit p38 by binding to the ATP pocket (Lee et al., 1994; Young et al., 1997). All imidazole based p38 inhibitors contain the elements of a 4-aryl-5-(pyridin-4-yl) imidazole (Gallagher et al., 1997) and among them SB203580 has been widely used to elucidate the roles of p38 in the

research. The effect of representative inhibitors on p38 activity is summarised in Table 1-4 (Lee et al., 1999).

The crystal structure and p38-mutant analysis demonstrate that beside the ATP-binding motif, the Thr106 residue is a major determinant for p38α/β inhibitor specificity. The His107, Leu108 and Met109 residues increase the binding affinity of imidazole based inhibitors (Gum et al., 1998; Lisnock et al., 1998; Tong et al., 1997). The substitution of the Thr106 residue of p38α by a Met residue abolishes the binding of the inhibitor and leads to SB-insensitive kinase, whereas the substitution of of JNK Met106 residue by a Thr residue confers it a SB-sensitivity (Gum et al., 1998; Eyers et al., 1999).

Table1-4: The p38 activity of representative inhibitors

Compound	p38 (IC_{50}, nM)
SKF86002	1500
SB203580	48
L-167307	5,0
Compound 1	83
SB220025	19
SB210313	1300
SB216385	480
RWJ68354	9,0

Binding of SB203580 to p38 blocks the activity of p38 but not its activation by MKK but by TAB1 (Ge et al., 2002). After anisomycin, sorbitol and sodium arsenite stimulation of Jurkat T, 3T3 or Hela cells, p38α is phosphorylated and activated. SB203580 also fails to block activation of p38 in arsenite activated 293 T cells, while it blocks the export of nuclear p38 to the cytoplasm (Ben-Levy et al., 1998). Nevertheless a decrease of p38α phosphorylation on its Tyr residue in the presence of 20 μM SB203580 after 45 min UV irradiation, anisomycin and sodium arsenite treatment has been observed (Lim et al., 1998).

At the concentration where p38α is inhibited, neither JNK nor ERK are blocked by SB203580 (Cuenda et al., 1995; Lim et al., 1998). A high level of selectivity for inhibition of p38 versus some protein kinases has been reported and is shown in table 1-5. The selectivity ratios based on IC_{50} often exceed 1000. SB203580 is equipotent against p38α and p38β. Other closely related MAPKs, such as p38γ, p38δ, JNK1 and ERK2 weakly inhibited if at all. The most potent inhibition reported for non-p38 MAPKs by SB203580 are c-Raf (IC_{50}=360) and JNK2β1(IC_{50}=280).

3.3 p38 MAPKs Upstream Activators: MKKs

Like other MAPKs, p38 MAPKs are activated when the Thr-Gly-Tyr motif which located between subdomains VII and VIII of the kinases are phosphorylated by dual kinases, the MAP kinase kinase (MKKs). Among the upstream kinases, MKK3 and MKK6 activate one or more of p38 isoforms. MKK3 has been shown to selectively activate p38α and p38γ (Enslen et al., 1998), while MKK6, which is 80% homologous to MKK3, activates all four isoforms (Goedert et al., 1997; Cuenda et al., 1997).

It is also reported that MKK4 activates both p38α and p38δ (Jiang et al., 1997) and MKK7 can activate p38δ (Hu et al., 1999). But it seems that MKK3 and MKK6 are likely to have important roles for the activation of p38 MAPKs under physiological conditions.

The substrate selectivity of MKKs may be a reason why each MKK has a distinct function.

The recombinant adenoviruses expressing activated MKK6bE are much more efficient than MKK3bE in inducing apoptosis of Jurkat T cell (Huang et al., 1997), while when expressed in cardiac myocytes, MKK3bE has more apoptotic effect (Wang et al, 1998).

Table 1-5: The sensitivity profile among protein kinases to SB203580

Kinase	Equivalent Residue	SB203580 (IC_{50}, nM)	Reference
p38α	T	48	Young et al., 1997
p38β	T	50	Kumar et al., 1997
p38γ	M	>10 000	Kumar et al., 1997
p38δ	M	>10 000	Kumar et al., 1997
JNK1	M	~5000	de Laszlo et al., 1998
JNK2β1	M	280	de Laszlo et al., 1998
JNK2α2	M	1900	de Laszlo et al., 1998
ERK2	Q	>100 000	Cuenda et al., 1995
PKB	S/T	500	Lali et al., 2000
MEK1	M	61 000	Cuenda et al., 1995
Cdc2	F	>50 000	Cuenda et al., 1995
TGFβI	S	20 000	Eyers et al., 1998
TGFβII	T	40 000	Eyers et al., 1998
c-Raf	T	360	de Laszlo et al., 1998
MK2	M	>10 000	Cuenda et al., 1995
LCK	T	20 000	Eyers et al., 1998

The selection of the appropriate MKKs varies with both the stimulus and the cell type. Normally the MKKs are activated in response to the cellular stresses and cytokines, but with some differences: MKK3 and MKK4 are preferentially activated by osmotic shock and anisomycin; while MKK6 is normally responsive to UV irradiation, osmotic shock, anisomycin and IL-1β (Meier et al., 1996). In rat PC12 and 293 cells, MKK3 and MKK6 phosphorylate p38α to the same extend whereas in KB cells, THP1 monocytes and rabbit skeletal muscle, MKK6 represents 95% of the p38α activator activity (Cuenda et al., 1996; Meier et al., 1996)

The names used for MKKs in different experimental systems are summarized in table 1-6.

Table 1-6: Names of MKKs

MKKs	**Other Names**
MKK1	MEK1
MKK2	MEK2
MKK3	SAPKK2, SKK2
MKK4	SAPKK1, SKK1, SEK1, JNKK
MKK5	MEK5
MKK6	SAPKK3, SKK3, MEK6
MKK7	SKK4

3.4 Further Upstream Activators

At least six further upstream activators capable of activating the MKK-p38 MAPK pathway *in vitro* or in co-transfection experiments have been identified, namely MAP kinase kinase kinases (MKKKs).

These include: (summarized in table 1-7)

MUK	MAPK kinase upstream kinase
MLK2/3	mixed lineage kinase2/3
MTK1	MAP three kinase 1
TAK1	TGFβ-activated protein kinase-1
ASK1	apoptosis signal regulating kinase 1

Normally, overexpression of these MKKKs leads to activation of both p38 and JNK pathways, that maybe the reason why p38 and JNK are often conactivated. There are different names of MKKKs in different experimental systems, they are summarized in the table 1-8.

The MKKKs are further diversified. For example, MTK1 is a major mediator of environmental stresses that activate the p38 MAPK pathway (osmotic shock, UV and anisomycin), but not cytokines (TNF) (Takekawa et al., 1997). Overexpression of ASK1 induced apoptotic cell death. ASK1 may be a key element in the mechanism of stress- and cytokine-induced apoptosis (Ichijo et al., 1997).

It has been reported that both small and large GTP-binding proteins are involved in the p38 signal cascade. Rac and Cdc42 are members of the Rho family of small GTP-binding proteins, they were identified as potential regulators of the p38 pathway (Zhang et al., 1995 ; Bagrodia et al., 1995). p21-activated kinases (Paks) are serine/threoinie protein kinases, the human homologues of Ste20. Pak can be activated by binding to its upstream regulators, Rac and Cdc42 (Bagrodia et al., 1995). Pak1 stimulates p38 activity. A dominant negative Pak1 suppresses both IL-1- and Rac/Cdc42-induced p38 activity (Zhang et al., 1995). MLK3 contains a Rac and Cdc42 binding motif, so Rac and Cdc42 may directly activate MLK3 (Tibbles et al., 1996).

Both fMet-Leu-Phe (FMLP) and platelet-activating factor (PAF) bind to the neutrophil cell surface via members of the seven trans-membrane spanning G-protein linked receptor family. Interaction between these chemokines and the G-protein-coupled receptors can lead to p38 pathway activation (Nick et al., 1997). FMLP strongly activates both p38 MAPK and ERK, while PAF preferentially activates p38 MAPK. The

activation of p38 MAPK by FMLP and PAF appears to occur through the activation of MKK3 (Nick et al., 1997).

Table 1-7: MKKKs of p38 pathway

MKKKs	Activated MKKs	Cell Type	Reference
MLK2	MKK3/6	KB cells, COS-1	Cuenda and Dorow, 1998
MTK1	MKK3/6/4	COS-7, Hela	Takekawa et al., 1997
DLK		COS-1 NIH3T3 cells	Hirai et al., 1997 Fan et al., 1996
MLK-3	MKK6		Tibbles et al., 1996
ASK1	MKK4/3/6		Ichijo et al., 1997
TAK1	MKK3/6 MKK6	COS7 Mv1Lu, C2C12	Moriguchi et al., 1996 Hanafusa et al., 1999

Table 1-8: MKKKs of p38 pathway and their other names

MKKKs	Other Names
MLK1	
MLK2	MST
MLK3	PTK, SPRK
MLK4	DLK(mouse), MUK(rat), ZPK(human)
MEKK1	
TAK1	
MTK1	SSK2/SSK22 (yeast)
ASK1	MKKK5

3.5 p38 MAP Kinases Inactivation

The magnitude and duration of activation of the MAPKs are properly regulated and have a great effect on determination of the fates and responses of cells. MAPKs are regulated by dual phosphorylation and dephosphorylation within the motifs TXY, respectively, by several upstream dual specificity kinases (MKKs) and several types of protein phosphatases.

MAP kinase inactivation is mediated by dephosphorylation via specific MAP kinase phosphatases (MKPs). There is an emerging family of dual specificity phosphatases acting on the MAPK superfamily. Eleven members of this group of dual specificity phosphatases have been reported, which are summarized in table 1-9.

The MKPs share sequence homology, but each has distinct properties concerning substrate specificity, tissue distribution, subcellular localization, and inducibility by extracellular stimuli. MKP-1, MKP-2, PAC1 and hVH3 localize in the nucleus, whereas MKP-3, MKP-4, and MKP-X localize in the cytoplasm; MKP-5 is distributed both in the cytoplasm and the nucleus (Table 1-9).

Among these MKPs, MKP1 inactivates several MAPKs, such as ERK, JNK and p38 (Sun et al., 1993). M3/6 is the first phosphatase of this family to display highly specific inactivation of JNK/SAPK and p38 MAP kinases. In contrast to M3/6, the dual specificity phosphatase MKP-3 is selective for inactivation of ERK family MAP kinases

Table 1-9: Members of MKPs

MKP	Other Names	Localization	Reference
MKP-1	3CH134/CL100	nucleus	Keyse and Emslie,1992
MKP-2	hVH2, TYP-1	nucleus	Guan and Butch, 1995 Misra-Press et al., 1995
MKP-3	Pyst1, rVH6	cytoplasm	Muda et al., 1996 Groom et al., 1996
MKP-4	Pyst3	cytoplasm	Muda et al., 1997
MKP-5		cytoplasm & nucleus	Tanoue et al., 1999
MKP-6			Marti et al., 2001
MKP-7		cytoplasm	Tanoue et al., 2001b
MKP-7		cytoplasm & nucleus	Masuda et al., 2001
MKP-X	Pyst2	cytoplasm	Muda et al., 1996 Groom et al., 1996
M3/6	hVH5		Muda et al., 1996b Theodosiou et al., 1996
VHR			Ishibashi et al,. 1992
PAC1		nucleus	Rohan et al., 1993
hVH3	B23	nucleus	Kwak et al., 1995 Ishibashi et al., 1994

(Muda et al, 1996b). Another dual specificity phosphatase, MKP 5, was reported to inactivate p38 and JNK (Tanoue et al., 1999). Recently, some new MKPs were reported to be found, MKP-6 (Marti et al., 2001) and two MKP-7 (Masuda et al., 2001; Tanoue et al., 2001b), they all inactivates MAP kinases. In COS-7 cells, expression of MKP-7 (Masuda-MKP-7) suppressed the activation mainly on JNK, p38 and ERK were also inactivated by MKP-7 (Masuda-MKP-7) (Masuda et al., 2001). Whereas Tanoue et al reported that their MKP-7 (Tanoue-MKP-7) is most similar to hVH5 in the primary sequence and predominantly localized in the cytoplasm when expressed in cultured cells. MKP-7 (Tanoue-MKP-7) possesses a NES sequence in the C-terminal stretch region, which regulates the subcellular localization of the molecule. MKP-7 (Tanoue-MKP-7) binds to and inactivates p38 MAPK and JNK, but not ERK.

Tanoue et al also proposed a tentative classification of MKPs according to the primary sequence of their MAPK-docking sites (Fig. 1-1) and they also found that MKPs have the substrate specificity toward the isoforms of the p38 MAPKs. CL100/MKP-1, MKP-5 and MKP-7 (Tanoue-MKP-7) selectively bind to and inactivate p38α and β, but not δ

or γ. Then, inactivation of p38 γ and δ in cells might be achieved by other phosphatases, such as serine/threonine protein phosphatases type 2C (PP2C) or protein tyrosine phosphatases (PTPs) (Tanoue et al, 2001b).

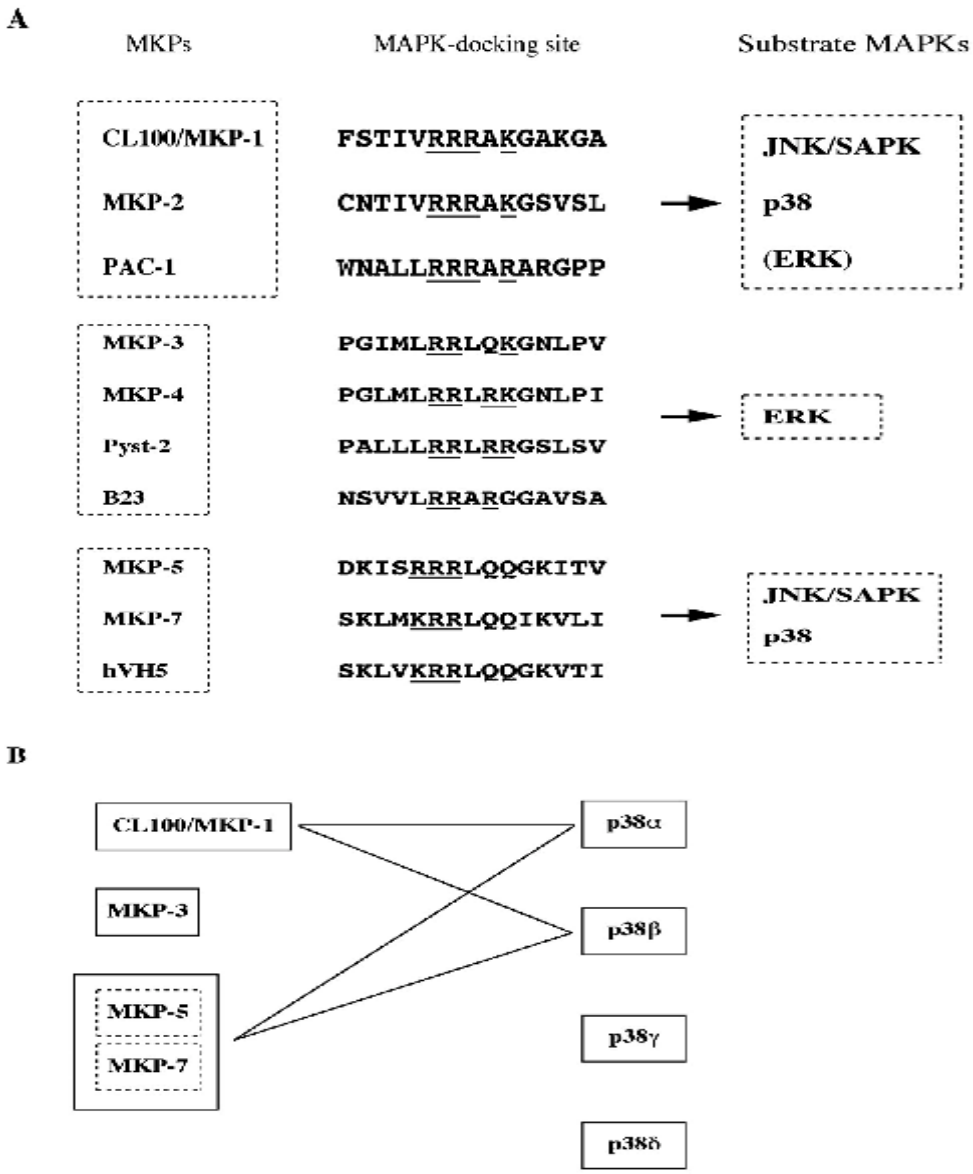


Fig. 1-1: The tentative classification of MKPs (Tanoue et al, 2001b): A, a proposed classification of MKPs according to the primary sequence of their MAPK-docking sites. The underlined amino acids are the positively charged amino acids which are supposed to be important for the docking interaction. The consensus sequence of each group is: XXRRRA(K/R) for the group containing CL100/MKP-1; XXXRRY(R/K)(Q)R/K(G) for the group containing MKP-3; X(K/R)RRLQQYK for the group containing MKP-7 (X is a hydrophobic residue, and Y is any residue except for K or R). B, MKPs show the substrate specificity toward the isoforms of the p38 MAPK family.

MKPs are potential negative regulators of MAPK cascades and as such are assumed to be involved in carcinogenesis by regulating cell proliferation and apoptosis. As a result, all human MKP genes have been mapped. Among them, MKP-2 and MKP-3 are mapped to a gene locus encoding tumor suppressors for prostate and pancreatic cancer, (Furukawa et al., 1998; Smith et al., 1997). MKP-X and MKP-5 are mapped to 3p21 (Smith et al., 1997) and to 1q41 (Masuda et al., 2000), respectively, where frequent deletions are reported in a number of different tumors. Masuda-MKP-7 is mapped to human chromosome 12p12 (Masuda et al., 2001) while Tanoue-MKP-7 gene is mapped to human chromosome 12 between the BAC clones, RPCI11-180M15 (accession number AC008115) and RP11-161A14 (accession number AC022276) (Tanoue et al., 2001b). Leptomycin B (LMB) is a specific inhibitor of nuclear export. MKP-7 is the first identified leptomycin B-sensitive shuttle MKP. Interestingly MKP-7 (Masuda-MKP-7) contains predicted functional motifs such as a NES and nuclear localization signals (NLSs), suggesting that it functions as a shuttle protein and a MAPK phosphatase (Masuda et al., 2001).

In addition to MKPs, protein tyrosine phosphatase (PTPase) and a serine/threonine protein phosphatase type 2C (PP2C) inactivated HOG1 pathway in yeast (Posas et al., 1996; Wurgler-Murphy et al., 1997). It is also reported that a human protein phosphatase, PP2Cα, negatively regulates the human stress-responsive p38 and JNK pathway. *In vivo* and *in vitro* studies indicate that PP2Cα dephosphorylates both MKK6, MKK4 and a p38 (Takekawa et al., 1998)

4. p38 MAPKs Targets: Downstream substrates of p38 MAP kinases

The main downstream substrates of p38 MAPKs are some protein kinases and transcription factors.

4.1 Protein Kinases

(1) MK2 and MK3/3pk

MK2 and MK3 have been identified as physiological substrates for p38 MAPK. p38α activated MK2 and this activation was inhibited by SB203580 *in vivo* ((Rouse et al., 1994). MK3 shares about 70% amino acid identity to MK2. MK3 was phosphorylated by both p38α and p38β. The activation was also blocked by SB203580 in Hela cells (McLaughlin et al., 1996). MK2 or MK3 is activated rather poorly by p38 γ and p38δ (Cuenda et al., 1997; Kumar et al., 1997).

The two isoforms of MK2 (63 kDa and 50 kDa) were described as ERK substrates first (Stokoe et al., 1992). Later, it was found that these two isoforms were coming from differential splicing of a 3.3 kb mRNA transcript (Stokoe et al., 1993). Mouse MK2 is activated by phosphorylation on Thr205 and Thr317 (Engel et al., 1995).
MK2 has a broad substrate specificity, phosphorylating proteins and peptides at serine residues that lie in Xaa-Xaa-Hyd-Xaa-Arg-Xaa-Xaa-Ser-Xaa-Xaa motif, where Hyd is a bulky hydrophobic residue (Phe > Leu > Val >> Ala) (Stokoe et al., 1993).

It has been found that under heat-shock conditions in mouse Ehrlich ascites tumor (EAT) cells and after treatment of human MO7 cells with tumor TNF-α, MK2 is activated. Further experiments showed that MK2 is responsible for phosphorylation of sHsps (Hsp25/27) both *in vitro* and *in vivo* (Engel et al., 1995b).

It has been reported that the activated MK2 and MK3 phosphorylate various relevant substrates, which including Hsp25/27; Lymphocyte-specific protein 1 (LSP1); CREB; ATF1; serum response factor (SRF); tyrosine hydroxylase; 5-lipoxygenase (5-LO); a basic helix-loop-helix (bHLH) transcription factor E47; and an ETS transcription factor ER81. These substrates are summarized in table 1-10.

The bHLH transcription factors E12 and E47 are encoded by the E2A gene and are generated by differential splicing of E12- and E47-specific bHLH-encoding exons. It is reported that MK3 and MK2 interact with E47 *in vivo* and are able to phosphorylate E47 *in vitro*, indicated that these kinases are regulators of E47 activity (Neufeld et al., 2000). The E47 amino acid sequence shows four potential minimal consensus motifs for phosphorylation by MKs, three in the extreme N terminus and one at Ser-529 (Sloan et al., 1996)

5-lipoxygenase (5-LO) catalyzes important steps in the synthesis of a group of inflammatory mediators, leukotrienes. It has been reported that 5-LO is a downstream target of MK2 and MK3 (Werz et al., 2000).

Table 1-10: Some substrates of MK2/3 and their phosphorylation residues

Substrate of MK2/3	**phosphorylation residues**	**Reference**
Hsp25 (murine)	Ser15, Ser86	Stokoe et al., 1992
Hsp27 (human)	Ser15, Ser78 and Ser82	Stokoe et al., 1992
LSP1	Ser204, Ser252	Huang et al., 1997
CREB	Ser133	Tan et al., 1996
ATF1	Ser63	Tan et al., 1996 Sun et al., 1996
SRF	Ser103	Heidenreich et al., 1999
tyrosine hydroxylase	Ser19	Thomas et al., 1997
5-LO		Werz et al., 2000
E47	more (such as 3 in the extreme N terminus and one at Ser529)	Neufeld et al., 2000 Sloan et a.l, 1996
ER81	Ser191, Ser216	Janknecht, 2001

ER81 is a ETS transcription factor and is identified as a novel target of MK2. By phosphorylating ER81 on Ser191 and Ser216, MK2 can inhibit ER81 transcriptional activity in a cell type-specific fashion (Janknecht, 2001). However, MK2 can also interfere with ER81-mediated transcription independently of serine 191 and serine 216 phosphorylation. (Janknecht, 2001).

MK2 ko mice were generated and embryonic fibroblast (MEF) cell line was established from the MK2 ko mice in our group (Kotlyarov et al., 1999). The MK2 ko mice show increased stress resistance and survive for LPS-induced endotoxic shock. This is due to a reduction of approximately 90% in the production of TNF-α and not to a change in signalling from the TNF receptor. It was also demonstrated that MK2 regulates biosynthesis of TNF-α at a post-transcriptional level (Kotlyarov et al., 1999). Using MK2 -/- MEF, it was found that MK2 was not involved in the phosphorylation of CREB and ATF1 (Rolli et al., 1999).

(2) MK5/PRAK

MK5 or PRAK (p38-regulated/activated kinase) was identified as serine/threonine kinase phosphorylated by p38 independently by two groups in mouse and human leading to the two different names (Ni et al., 1998; New et al., 1998). Meanwhile, it is highly probable that PRAK is the human homologues of murine MK5. MK5 and PRAK are compared in table 1-11.

PRAK, like MK2, phosphorylates Hsp27 at the functionally relevant sites (New et al., 1998). And it was suggested that p38α and p38β are *in vivo* regulators of PRAK (New et al., 1998). Although MK5/PRAK was found three years ago, we still know very less about this kinase.

Table 1-11: MK5 and PRAK

	MK5	**PRAK**
origin	murine	human
amino acid	473	471
molecular mass	54 kDa	54 kDa
tissue distribution	heart, brain, lung, liver, skeletal muscle, kidney, testis	heart, brain, lung, liver, skeletal muscle, kidney placenta, pancreas
cell line distribution		293, A549, Hela, Jurkat, HepG2
mRNA	2.3 kb	2.4 kb
stimuli		arsenite anisomycin H_2O_2 PMA Calcium ionophore (A23187) TNF-α
be phosphorylated by	ERK and p38	p38α and p38β
activated by	ERK and p38(*in vitro*)	MKK6(E), p38α and p38β
relevant site	Thr182	Thr182
LXTP motif	LMTP	LMTP
phosphorylates		Hsp27/25 (*in vitro* and *in vivo*)
does not phosphorylate		GST-c-Jun GST-ATF2 PHAS-1 MBP-1
Reference	Ni et al., 1998	New et al., 1998

(3) MAPK interaction protein kinase 1 (MNK1)

MNK1 and MNK2 are structurally similar to MK2 and MK3. MNK1 binds tightly to ERK1 and p38, whereas MNK2 only binds to ERK1/2 (Waskiewicz et al., 1997). Both ERK and p38 phosphorylate and activate MNK1 in response to peptide growth factors, phorbol esters and environmental stresses. The activation by these stimuli is differentially inhibited by PD98059 and SB202190 (Fukunaga and Hunter, 1997). It has been found that activated MNK1 phophorylates eIF-4E at the physiologicall relevant site Ser209 both *in vitro* (Waskiewicz et al., 1997; Wang et al, 1998b) and *in vivo* (Knauf et al., 2001). The phosphorylation of eIF4E via MNK1 is mediated via the activation of either the ERK or p38 pathway and the kinase activity of MNKs, eventually through phosphorylation of eIF4E, may serve to limit cap-dependent translation under physiological conditions (Knauf et al., 2001).

(4) MSK1

MSK1 is a protein serine/threonine kinase that contains two protein kinase domains in a single polypeptide. Like MNK1, MSK1 is activated both *in vitro* and *in vivo* by either ERKs or p38, it is activated by both stress and mitogen (Deak et al., 1998; New et al., 1999). In HeLa, PC12 and SK-N-MC cells, PD 98059 and SB 203580 are both required to suppress the activation of MSK1 by TNF, NGF and FGF. MSK1 is localized in the nucleus of unstimulated or stimulated cells, and phosphorylates CREB at Ser133 with a Km value far lower than PKA, MK1 and MK2. This suggests that MSK1 may mediate the growth-factor and stress-induced activation of CREB (Deak et al., 1998).

MSK1 also can phosphorylate histone 2B, histone 1, histone H3 and HMG-14 *in vitro* (New et al., 1999; Thomson et al., 1999). The phosphorylation of histone H3 on serine 10 and HMG-14 on serine 6 would induce the immediate-early (IE) gene expression in response to a wide variety of stimuli. This induction can be blocked by the protein kinase inhibitor HB89, PD098059 or SB203580. MSK1 may mediate the nucleosomal response through ERK and p38 pathway, but not JNK (Thomson et al., 1999).

MSK1, MSK2 and double knockouts (both MSK1 and MSK2) mice were produced recently. Experiments using MEFs derived from these ko mice showed that MSK1 and MSK2 are required for the stress-induced phosphorylation of CREB and ATF1. The knockout of both MSK1 and MSK2 resulted in a 50% reduction in c-fos and junB gene transcription in response to anisomycin or UV radiation but only a small reduction in response to TPA or EGF in fibroblasts. The transcription of egr1 in response to both mitogenic and stress stimuli, as well as stress-induced apoptosis, was unaffected in the MSK1/MSK2 double knockout (Wiggin et al., 2002).

4.2 Transcription Factors

Several transcription factors can be phosphorlyated and activated by p38 pathway. And many of the transcriptional events stimulated by p38 MAPK may also be mediated by the activation of the protein kinases which are p38 downstream direct targets, such as MK2, MNK1 and MSK1.

(1) ATF2

ATF2 is a member of the ATF/CREM family. All members of this family contain a DNA binding domain consisting of a cluster of basic amino acids and a leucine zipper region, the so called b-ZIP (Busch and Sassone-Corsi, 1990). They form homodimers or heterodimers through their leucine zipper regions and bind to CRE. p38β has been proposed to phosphorlyate ATF2 with higher efficiency than p38α (Jiang et al., 1997; Stein et al., 1997), while others have shown that all four isoforms phosphorlyate ATF2, as well as Elk1 and SAP1, with equal efficiency (Cuenda et al., 1997; Goedert et al., 1997; Kumar et al., 1997). p38 MAPKs and JNK phosphorylated ATF2 at Ser90, as well as at Thr69 and Thr71 (Cuenda et al., 1997). It has been shown that p38 activated ATF2 was involved in the TNFR1-induced gene expression of TNF-α (Brinkman et al., 1999) and might play an important role in the TGF-β signalling via TAK1 and p38 pathway (Sano et al., 1999).

Using GST-ATF2-(1-115), the kinetic mechanism for the dual phosphorylation has been studied. It has been found that the dual phosphorylation of the two residues within GST-

ATF2-(1-115), Thr-69 and Thr-71, occurs by a two-step mechanism where p38 dissociates from the protein after each phosphorylation event. The kinetics of this process is predicted to have important implications for the activation of ATF2 by p38 *in vivo* and highlights the potential that two-step distributive mechanisms have for controlling the amplitude sensitivity of signal transduction pathways (Waas et al., 2001).

(2) myocyte enhancer factor 2C and 2A (MEF 2C and 2A)

The MEF2 family is composed of four members: MEF2A, MEF2B, MEF2C, and MEF2D. Members of the MEF2 family of transcription factors bind as homo- and heterodimers to the MEF2 site found in the promoter regions of numerous muscle-specific, growth- or stress-induced genes (Han et al., 1997b). MEF2A and MEF2C exhibit about 56% identity sequence conservation throughout their lengths (Yang et al., 1999). MEF2A and MEF2C have been shown to be phosphorlyated and activated by p38 MAPKs (Han et al., 1997b; Zhao et al., 1999). Transcriptional activation *in vivo* of MEF2A and MEF2C by p38α and p38β requires a kinase docking domain, D-domain (Yang et al., 1999). The Thr312 and Thr319 residues of MEF2A and the Ser287, Thr293 and Thr300 residues of MEF2C are necessary for the activation of p38 MAPKs (Han et al., 1997b; Zhao et al., 1999). With MEF2C, selective phosphorlylation of residue Thr293 is a tissue-specific activating signal in differentiating myocytes (Wu et al., 2000). Among the four p38 isoforms, p38α is the most potent kinase for MEF2A (Zhao et al., 1999).

In monocytic cells, LPS increases the transactivation activity of MEF2C through p38-catalysed phosphorylation. Activation of MEF2C results in increased c-jun gene transcription. This suggested that p38 may influence host defence and inflammation by regulating c-Jun production (Han et al., 1997b). It has been shown that the p38 MAPK pathway promotes skeletal muscle differentiation at least in part via activation of MEF2C (Zetser et al., 1999; Wu et al., 2000).

(3) ternary complex factor (TCF) Proteins: Elk1 and SAP1

TCFs are a subgroup of Ets-domain transcription factor family. Elk1 and SRF accessory protein 1 (SAP1) are members of the TCF family.

Elk1 binds to Ets sites in the pip92 (IE gene) promoter (Latinkic and Lau, 1994). It has been shown that Elk1 was involved in the pip92 expression during anisomycin-induced cell death in fibroblast NIH3T3 cells (Chung et al., 2000). JNK, p38 MAPK and ERK all can phosphorylate Elk1 (Janknecht and Hunter, 1997; Whitmarsh et al., 1997), and high levels of JNK and p38 activities have been correlated with the induction of apoptosis in many instances (Xia et al., 1995; Chen et al., 1996; Chung et al., 2000).

It has been reported that Elk-1 is barely activated by p38, most likely because the critical residues Ser383 and Ser389 are poorly phosphorylated by p38 MAP kinase. In contrast, SAP1 is efficiently phosphorylated by p38 MAP kinase on residues Ser381 and Ser387 (Janknecht and Hunter, 1997). SAP1 contains two domains which specify substrate phosphorylation. The D-domain serves to promote selective targeting of ERK2, p38α, and p38β; whereas the second domain, FXF motif promotes targeting by ERK2 and, to a lesser extent, p38α, but not p38β. The FXF motif is conserved between SAP1 and Elk1 (Galanis et al., 2001).

SAP1 could be an important target for mitogens, stress and apoptotic signals to elicit a nuclear response (Janknecht and Hunter, 1997).

(4) C/EBP(CCAAT/enhancer-binding protein) Family: CHOP (cAMP response element-binding protein-homologous protein/growth arrest DNA damage 153 (CHOP/GADD153)) and C/EBPβ

Some of C/EBP family of transcription factors have been reported to be controlled by p38 MAPK. CHOP is a member of the C/EBP family of transcription factors and accumulates under conditions of stress and mediates effects of cellular stress on growth and differentiation (Wang and Ron, 1996). In response to stress, CHOP undergoes phosphorylation *in vivo* on two adjacent serine residues 78 and 81, which is blocked by SB203580. Phosphorylation of CHOP positively correlates with its transcription activity. CHOP thus serves as a link between p38 and cellular growth and differentiation (Wang and Ron, 1996).

Another member of C/EBP family, C/EBPβ, is a substrate of p38. In 3T3-L1 fibroblasts, inactivation of p38 during only the initial stages of differentiation can prevent conversion of the fibroblasts to adipocytes (Engelman et al., 1998).

4.3 Others

(1) cytosolic phospholipase A2 (cPLA2)

cPLA2 is responsible for thrombin-stimulated mobilization of arachidonic acid for the synthesis of thromboxane A2 in human platelets. It has been shown that cPLA2 is phosphorylated and activated p38α, so cPLA2 is thought to be a physiological target of p38α (Kramer et al., 1996). Ser505 and Ser727 of cPLA2 could be phosphorylated during its activation. Interestingly, it has been found that Ser505 is phosphorylated by p38 protein kinase, mainly by p38α; whereas Ser727 is phosphorlyated not by p38, but by a p38 direct downstream target protein kinase, such as MNK1 or a closely related protein kinase (Hefner et al., 2000).

(2) Stathmin/Op18

Stathmin, a cytoplasmic protein that is linked to regulation of microtubule dynamics and is a substrate of several intracellular signalling kinases, was also identified to be a substrate of p38δ in a solution kinase assay. Osmotic stress activates p38δ has been shown to phosphorylate stathmin on Ser25 and Ser38 both *in vitro* and in cells (Parker et al., 1998). It has been reported that TNF-induced microtubule stabilization is mediated by hyperphosphorylation of oncoprotein 18 (Op18, stathmin) and that this promotes cell death in the mouse fibrosarcoma cell line L929 (Vancompernolle et al., 2000).

5. Subcellular Localization of p38 MAPKs and Their Downstream Targets

Subcellular localization of p38 MAPK and their downstream targets or the nuclear-cytoplasmic transport of p38 MAPKs may show their different physiological functions in certain conditions. In different cells and in different experimental systems, the subcellular localization may also be different.

Immunofluorescence microscopy demonstrated that over-expressed p38 MAP kinase is present in both the nucleus and cytoplasm. Activation of the signalling cascade by UV irradiation does not cause marked redistribution of p38 MAP kinase from the cytoplasm to the nucleus in COS cells (Raingeaud et al., 1995). In transfected cells, GFP-MK2 is located predominantly in the nucleus. Upon stress, it rapidly translocates to the cytoplasm and the translocation is blocked by SB203580 and leptomycin B (Engel K et al, 1998). Following phosphorylation of MK2, nuclear p38 is exported to the cytoplasm in a complex with MK2. The cytoplasm translocation of MK2 requires phosphorylation by p38 without a requirement for MK2 activity (Ben-Levy et al., 1998). MK2 serves both as an effector of p38 by phosphorylating substrates and as a determinant of cellular localization of p38. Nuclear export of p38 and MK2 may permit them to phosphorylate cytoplasmic substrates, such as eukaryotic initiation factor (eIE)-4E and PHAS-1.

Both endogenous and GFP-MK5 are in nuclear in resting cells. Like MK2, MK5 also has a functional NLS and a NES. Treatment of cells with arsenite results in nuclear export of MK5. Time studies showed that this transport was much slower then MK2. Sorbitol induced nuclear export of MK2, but not of MK5. Arsenite-induced export of MK5 is inhibited by leptomycin B (Prof. Ugo Moens and Dr. Ole Morten Seternes' observation, unpublished). Transfection of GFP-MK5 or co-transfection of GFP-MK5 and p38 in Hela cells, after stimulation of arsenite, we checked the export of MK5 and got the comparable results.

6. p38 Pathway Functions

6.1 Cytokines: Production and Gene Expression

(1) IL-1β and TNF-α

Synthetic MALP-2 can activate p38 and the activation of p38 induces mRNA synthesis and protein secretion of IL-1β and TNF-α in human monocytes/macrophages and the murine macrophage cell line RAW 264.7 (Garcia et al., 1998). Experiment with MK2 ko mice showed that p38 through the activation of MK2, controls IFN-γ and TNF-α expression in spleen cells at a post-transcriptional level (Kotlyarov et al., 1999). It has also been reported that inhibition of p38 MAPK pathway by SB203580 reduces LPS-induced TNF-α production not only by affecting the translation level, but also by destabilizing TNF-α mRNA. SB203580 could reduce more than 50% of TNF-α mRNA expression in differentiated THP-1 cells and almost 80% in human blood monocytes (Rutault et al., 2001).

(2) IL-4

Using purified human $CD4^+$ peripheral blood T cells, it has been shown that CD28 stimulation alone activates p38α. SB 203580 blocked CD28-induced cell proliferation as well as CD3/CD28-induced IL-4 production, largely at the mRNA level (Schafer et al., 1999).

(3) IL-5

IL-5 synthesis is dependent on p38 MAPK activity and SB203580 inhibited IL-5 synthesis, suppressed IL-5 mRNA expression in human Th cells (Mori et al., 1999).

(4) IL-6

IL-1β is a potent activator of IL-6 synthesis in human fibroblast-like synoviocytes (FLSs) (Guerne et al., 1989). In response to IL-1β stimulation in human FLSs, p38 was activated and was found to be involved in IL-6 synthesis at the transcription level by stabilizing IL-6 mRNA, without affecting the rate of IL-6 gene transcription (Miyazawa et al., 1998). Using of SK&F86002, an cytokine-suppressant anti-inflammatory drug (CSAID), p38 was found also being involved in TNF-α -induced IL-6 production in human osteoblastic cells (SaOS2) (Blanque et al., 1997). In MK2 ko mice, IL-6 mRNA level is greatly reduced compared to the wild type (Kotlyarov et al., 1999).

(5) IL-8

Mycoplasma fermentans lipid-associated membrane proteins (LAMPf) induce the production of high levels of IL-8 by THP-1 and PMN cells at the same extent as LPS, and both ERK1/2 and p38 cascades play a key role in the production of IL-8 (Marie et al., 1999). It is also reported that MKK6 pathway contributes to induction of IL-8 synthesis by stabilizing its mRNA (Holtmann et al., 1999).

(6) IL-12

MKK3 ko macrophages have a deficit in LPS-induced p38 MAPK activation, in LPS- and CD40-induced production of IL-12, and in LPS-induced production of IL-1, these cytokines production are still SB203580 sensitive. IFN-γ production following immunization with protein antigens and *in vitro* differentiation of naive T cells is greatly reduced. RNAse protection assays show that only IL-12 mRNA level is decreased in LPS-treated macrophages. Report assays confirm that regulation of IL-12 production is at least partly at the transcriptional level (Lu et al., 1999).

So, p38 pathway regulates cytokines gene expression and controls cytokines production. Mostly these regulations are on the transcriptional level, while IFN-γ and TNF-α are also affected at the post-transcriptional level.

6.2 Other Gene Expressions

(1) IE Genes

p38 MAPK pathway regulates some immediate early gene expression. Activation of the p38 signalling pathway has been linked to activation of Elk-1 ternary complex factor, which binds to the c-fos promoter (Whitmarsh et al., 1997). In monocytic cells, LPS increases the transactivation activity of MEF2C through p38-catalysed phosphorylation. Activation of MEF2C increased c-jun gene transcription. This indicated that p38 may influence host defence and inflammation by maintaining the balance of c-Jun protein consumed during infection (Han et al., 1997b).It has been also reported that GnRH-induced p38 MAPK activation may selectively contribute to the regulation of c-fos protooncogene expression in αT3–1 cells (Roberson et al., 1999). Echovirus 1 (EV1) infection increases the mRNA levels of cellular IE genes in host cells. p38 was the main inducer of junB expression, whereas both p38 and ERK pathways were involved in the induction of c-fos (Huttunen et al., 1998).

(2) VCAM-1: (endothelial adhesion molecule vascular cell adhesion molecule-1)

In human umbilical vein endothelial cells, SB203580 suppressed the TNF-α-induced surface expression of the endothelial adhesion molecule vascular cell adhesion molecule (VCAM)-1. The VCAM-1 mRNA accumulation was not affected by SB203580, this suggested that the p38 MAPK pathway regulates the expression of VCAM-1 at the post-transcriptional level (Pietersma et al., 1997).

(3) iNOS: (inducible nitric oxide synthase)

The iNOS converts the L-arginine to L-citrulline and nitric oxide (NO). NO production is directly correlated with iNOS activity and its expression. The promoter region of iNOS has been characterized in murine with RAW 246.7 macrophage cell line. Sequence analysis showed that this region contains several transcription factor binding sites: NF-κB, AP-1, IFN-γ response elements(γ-IRE), γ-activated site (GAS), IFN-γ stimulated response element (ISRE), TNF-α responsive element, among others (Xie et al., 1993; Lowenstein et al., 1993; Xie et al., 1994; Nathan et al., 1994). Reporter assay showed that AP-1 motif is not implicated in LPS-induced iNOS expression, but κB motif is. After stimulation, NF-κB translocates to the nucleus and is bound to the promoter of iNOS in rat hepatocytes in primary culture (Diaz-Guerra et al., 1996).

TNF-α plus IFNγ elicited a strong enhancement of nitric oxide synthesis. Western blot- and RT-PCR-experiments showed that these two cytokines together induce the expression of iNOS. The induction of iNOS could be inhibited by SB203580. This suggested that p38 pathway may play an important role in regulating the induction of iNOS gene expression in cytokine-treated cells (Bhat et al., 1999).

Full activation of the human iNOS (hiNOS) promoter by cytokines required downstream and upstream NF-κB and AP-1 transcription factor binding sites. The combination of LPS and IFN-γ, but neither alone, increased hiNOS promoter activity 28-fold. p38 MAPK and ERK pathways by regulating the binding of AP-1 to specific promoter sequences activate the promoter of hiNOS (Kristof et al., 2001).

(4) COX-2: (cyclooxygenase-2)

COX-2 converts arachidonic acid to the endoperoxide intermadiate PGH2, which is subsequently converted to prostaglandin (PEG2) by the action of cell specific synthetases. In rat renal mesangial cells, IL-1β-induced COX-2 expression and PEG2 production depends on both JNK and p38 MAPK (Guan et al., 1998)

LPS induced the expression of COX-2 protein and COX-2 mRNA as well as the phosphorylation and activation of ERK2 and p38 MAPK in monocytes. Since the induction of COX-2 mRNA and COX-2 protein was inhibited by the specific inhibitors of ERK and p38 MAPK, suggesting that the activation of ERK2 and p38 MAPK is involved in COX-2 expression (Niiro et al., 1998).

It has also been found that p38 MAPK cascade plays a role in the transcription and stabilization of COX-2 mRNA. IL-1 stimulated COX-2 transcription about 2-fold in HeLa cells. In the cells previously stimulated with IL-1, SB 203580 treatment caused rapid destabilisation of COX-2 mRNA (Ridley et al., 1998). Further experiments demonstrated that p38 MAPKs were essential for stabilizing COX-2 mRNA in human monocytes: when cells stimulated for 4 h with LPS were treated with actinomycin D,

COX-2 mRNA decayed slowly. Treatment of stimulated cells with 2 μM SB 203580 caused a rapid disappearance of COX-2 mRNA, even with actinomycin D present (Dean et al, 1999). Other components of p38 MAPK cascade, MKK6, MK2 and maybe Hsp27, were also found to be involved in the stabilization of COX-2 mRNA in Hela cells (Lasa et al., 2000). Further experiment showed that Dexamethasone destabilizes COX-2 mRNA by inhibiting p38 MAPK (Lasa et al., 2001).

6.3 Apoptosis

Apoptosis is a common cellular response to stress and ample evidence showed that activation of p38 pathway correlated with apoptosis. p38 pathway has been shown to be involved in cell death induced by NGF withdrawal in PC12 cells (Xia et al., 1995; Kummer JL et al, 1997), by ceramide in U937 and BAE cells (Verheij et al., 1996), by anti-IgM antibody in human B lymphocytes (Graves et al., 1996), and by Fas-ligation in Jurkat cells (Juo et al., 1997). In cultured chick fetal forebrain neurons, p38 activity was down-regulated by insulin, which can support survival of these cells (Heidenreich and Kummer, 1996). It has also been reported that thrombospondin-1 (Jimenez et al., 2000), oxidative stress, TNF-α and TL-1, a TNF-like cytokine (Yue et al., 1999), induce a p38-dependent apoptosis or signalling in endothelial cells.

Vascular endothelial growth factor (VEGF) is capable of eliciting biochemical responses that either enhance cell survival by PI3-kinase/Akt pathway or might lead to cell death by p38 MAPK pathway. Blockade of PI 3-kinase or Akt signalling attenuates VEGF-stimulated MEKK3 phosphorylation and increases p38 signalling, thus enhancing apoptosis. On the other hand, overexpression of constitutively active Akt enhances MEKK3 phosphorylation and down-regulates p38 activation (Gratton et al., 2001). Recently it has been reported that ERK-1/2 and p38 MAPK oppositely regulate NO-induced apoptosis of chondrocytes. NO-induced p38 MAPK functions as an induction signal for apoptosis and in the maintenance of chondrocyte phenotype, whereas ERK activity causes dedifferentiation and operates as an anti-apoptotic signal (Kim et al., 2002).

Though p38α is activated in Jurkat T cells during Fas ligation, its activity is not required for apoptosis (Juo P et al, 1997). While other evidence showed that SB203580 do block the apoptosis in serum depletion induced Rat-1 cell death (Kummer et al., 1997), NGF withdrawal-induced PC12 cell apoptosis (Kummer et al., 1997) and TL-1-induced bovine pulmonary artery endothelial cells apoptosis (Yue et al., 1999). So the role of p38 MAPK pathway in apoptosis is cell type- and stimulus- dependent.

6.4 Cell Growth and Differentiation

p38 pathway is also involved in controlling cell growth and differentiation. Cdc42Hs, a member of Rho family, activated p38α in NIH-3T3 cells. The activation of p38α can inhibit serum-stimulated cell cycle progression at G1/S transition point (Molnar et al., 1997). The p38α was activated in mammalian cultured cells when the cells were arrested in M phase by disruption of the spindle with nocodazole (Takenaka et al., 1998).

p38 MAPK pathway plays an important role in cell differentiation in several cell types. The differentiation of PC12 cells into neurons (Morooka et al., 1998) and 3T3-L1 cells into adipocytes (Engelman et al., 1998) both need p38α and or p38β, since SB203580

can inhibit the effect. It has been found that p38 MAPK pathway is involved in regulating chondrocyte differentiation. Parathyroid hormone (PTH) inhibites p38 MAPK and the inhibition converts a hypertrophic cell phenotype to a prehypertrophic one (Zhen et al., 2001).

Table 1-12: Some cell lines mentioned in the introduction

Cell line	**Cell type or origin**
RAW 264.7	murine macrophage cell line
C2C12	muscle cell line
CT6	murine cytokine-dependent T cell line
L929	mouse fibrosarcoma cell line
MEF	mouse embryonic fibroblast
EAT	mouse Ehrlich ascites tumor cells
NIH3T3 cells	fibroblast
3T3-L1	fibroblast
SK-N-MC cells	fibroblast
H19-7	hippocampal progenitor cells
THP-1	human monocytic cell line
Mono Mac 6 cells	human monocytic cell line
HL-60 cell line	human promyelocytic leukemic
PMNs	human polymorphonuclear neutrophils
Hela	human epithelial cell
HEK293	human embryonic kidney cell line 293
SH-SY5Y	human neuroblastoma cells
A549	human lung epithelial cells
HUVEC	human umbilical vein endothelial cells
SaOS2	human osteoblastic cells
BPAEC	bovine pulmonary artery endothelial cells
PC12	rat phaeochromocytoma cell line
COS	green monkey kidney cells

Fibroblast growth factor (FGF)-2 induces bovine capillary endothelial cells to form tubular growth-arrested structures in collagen gel cultures. Recently it has been reported that p38 MAPK negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis (Matsumoto et al., 2002). SB202190 can enhance FGF-2–induced tubular morphogenesis by decreasing apoptosis, increasing DNA synthesis and cell proliferation, and enhancing cell differentiation. Whereas overexpression of dominant negative mutants of the MKK3 and MKK6 also supported FGF-2–induced tubular morphogenesis (Matsumoto et al., 2002).

II. MATERIALS and METHODS

1. Materials

The cell lines, mice, plasmid constructs, antibodies, oligonucleotides and some chemical regents and equipments used in this study are listed in the tables below.

Table 2-1: Cell lines and mouse strains

Cell line/ mouse strain	Description	Reference or source
MEF wt	mouse embryonic fibroblast	prepared by our group
MEF MK2 -/-	MK2 deficient mouse embryonic fibroblast	prepared by our group
MEF MK5-/-	MK5 deficient mouse embryonic fibroblast	prepared by our group
Hela cell	human epithelial cell	our group
ES cell	E14-1 ES cell line	provided by Prof. Dr. Carmen Birchmeier
MK 5 Chimeras		EMBL Heidelberg
C57BL/H199		Charles River

Table 2-2: Regents for cell culture

Regent	Description	Reference or source
DMEM	Dulbecco's l modified Eagle's medium	GibcoBRL, 31966-021
NEAA	nonessential amino acids	GibcoBRL, 1140035
FBS	fetal bovine serum	Biochrom, S0115
FBS	fetal bovine serum (especially for ES cells)	Sigma, F7524 (Lot. No. 77H3356)
T/E	Trypsin/EDTA	GibcoBRL, 25300-054
pen/strep	penicillin/streptomycin	GibcoBRL, 05140-114
Lif (ESGRO)		prepared by our group
50 mM ß-ME	2-Mercaptoethanol	GibcoBRL, 31350-010
Mitomycin C		Sigma, M0503
G-418 sulate	Geneticin	Calbiochem, CN345810
Gelatin		Sigma, G9391

Table 2-3: Plasmids

Plasmid	Description	Reference or source
MK5 genomic DNA	from the mouse 129 inbred strain	Genome Systems
pBlueScript KS+		Stratagene
pSG5 vector		Stratagene
pcDNA3		Invitrogen
pREP8		Invitrogen
ptV-O	"neo" vector	provided by Prof. Dr. Carmen Birchmeier
pSG5-myc-MK5-wt	wt MK5 expression vector	provided by Dr. Ole Morten Seternes
pcDNA4--His-Max-MK5-ko	ko MK5 expression vector	our group
pEGFP-MK5		provided by Dr. Ole Morten Seternes
pEGFP-MK2		our group
pcDNA3-HA-p38		our group

The oligonucleotides used in the experiments were all purchased from MWG.

Table 2-4: Oligonucleotides and their sequences

Oligos	Sequence (5'------3')	Description
		Relevant sequence of MK5 gene (see result, Fig 3-3)
MK5-RS-1833d	cgtaacactagccacagttgtaactga	RS 1833-1859, 5' primer
MK5-RS-2272d	gtaggccagcactgtgccaactgatgta	RS 2272-2299, 5' primer
MK5-RS-2830rc	catatacttgtaagcacagctctgagtt	RS 2830-2803, 3' primer
		MK5 cDNA (AF039840)
MK5-1d	agttgaccaaggtgatttgatgaca	MK5 cDNA 1228-1252, 5' primer
MK5-2d	ggagaagtctggcatcatacctacctc	MK5 cDNA 1315-1341, 5' primer
MK5-1rc	ctggctccactcttcttctgggaactc	MK5 cDNA 1522-1496, 3' primer
MK5-2rc	gggatggtccaacactccctcgat	MK5 cDNA 1612-1589, 3' primer
MK5-707d	atgtcggaggacagcgacatggagaaag	MK5 cDNA 707-235, 5' primer
MK5-988d	cagggctcgactcttaattgtaatgg	MK5 cDNA 988-1013, 5' primer
MK5-1189rc	atctgcttttcaaggataactctctg	MK5 cDNA 1189-1164, 3' primer (in the delete region)
MK5-1290rc	agtacctgaggtgctacatagtaagg	MK5 cDNA 1290-1265, 3' primer
MK5-2128rc	ctactggggctcgtggggaagggtctgc	MK5 cDNA 2128-2100, 3 primer
		ß-actin cDNA (X03672)
ß-actin-200d	ccagggtgtgatggtgggaatg	ß-actin cDNA 200-221, 5' primer
ß-actin-709rc	cgcacgatttccctctcagctg	ß-actin cDNA 709-688, 3' primer

Table 2-5: Probes for hybridization

Probe	Description	Reference or Source
P1	external probe	Bam HI-Sac I fragment, 1 kb
P2	internal probe	Sac I fragment, 2.2 kb
P3	neo probe	neo box, 1.2 kb
P4	internal probe	Spe I-Bam HI fragment, 2 kb
P5	for NB	MK5 CDS, cDNA 707-2128

Table 2-6: Antibodies, enzymes and chemicals

Ab/enzyme/Chemical	Description	Reference or Source
Anti-PRAK		Upstate Biotechnology, 06960
PRAK substrate peptide		Upstate Biotechnology, 12-136
Anti-MK2		prepared by our group
Hyperfilm ECL	for WB	Amersham, RPN3103H
Hybond ECL	nitrocellulose membranes for WB	Amersham, RPN303D
Rapid-hyb buffer	for SB	Amersham, RPN1636
[α–^{32}P]-dATP		NEN, NEG512H
[α–^{33}P]-dATP		ICN, 58200.2
[γ–^{33}P]-ATP		ICN, 58404.2
p81 paper	for kinase assay	Whatman, 3698-915
Proteinase K		Sigma, 2308
Restriction enzymes		New England Biolabs
Taq DNA polymerase	PCR	GibcoBRL, 11508-017
T4 DNA ligase	Cloning	GibcoBRL, 15224-017
Protease inhibitor cocktail	kinase assay	Roche, 1836170
SB203580		Calbiochem-Novabiochem, 538944
D-Galactosamine		Sigma, G0264
Lipopolysaccharides (LPS)		Sigma, L8274
L-Histidinol		Sigma, H6647

Table 2-7: Kits

Kit	Description	Reference or source
Plasmid Maxi Kit		QIAGEN, 12161
HotStarTaq polymerase	PCR kit	QIAGEN, 203205
QIAEX II gel extraction kit		QIAGEN, 20021
RNeasy mini kit		QIAGEN, 74104
peqGOLD RNAPure FL™	extract RNA from cells	peqLab, 30-1110
display THERMO-RT	RT kit	Display System Biotech
TOPO TA cloning kit		Invitrogen, k4500-01
LipofectAMINE PLUS	transfection kit	GibcoBRL, 10964-013
Megaprime DNA labelling system	probe labelling kit	Amersham, RPN1604
Cell Phect transfection kit		Amersham, 27-9268-01
Bio-Rad Protein Assay	protein concentration	Bio-Rad, 500-0006
Mouse Immunoglobulin Panel Clonotyping System-HRP		Southern Biotechnology Associates, 5300-01 Southern Biotechnology Associates, 5300-05
ELISA kit (Quantikine™)	measurement of cytokines	R&D Systems

Table 2-8: Equipments

Equipment	Description	Reference or source
Biometra TRIO-Themroblock	PCR Machine	Biotron (Germany)
CO_2 UNITHERM 150 Hybridzer	Incubator	UNIEOUIP
Bio-Rad Gene Pulser II	Electroporation machine	Bio-Rad
FUJIFILM BAS 1500	phosphoimager	

2. Methods

2.1 Construction of targeting vectors

2.1.1 Isolation of MK 5 genomic DNA

P1 Clone (P1+MK5 genomic DNA) was purchased from "Genome Systems" . The plasmid was transferred from NS3529 to NS3516 via transduction and the DNA was isolated according to the "P1 Manual".

The MK5 genomic DNA and ES cell DNA were examined by PCR with primers, MK5-1d, MK5-1 rc, MK5-2d and MK5-2 rc. 0.5 µg of MK5 genomic DNA or ES cell DNA was used as a template to perform PCR in a 50 µl reaction volume: 1 × PCR buffer, 0.2 mM each dNTP, 0.2 µM Primers (1d+1rc; 1d+2rc; 2d+1rc or 2d+2rc), 2.5 units of Taq DNA polymerase (GibcoBRL) with thermal-cycles including the first denaturation at 96 ^{o}C for 3 min, 35 cycles of 96 ^{o}C for 1 min, 60 ^{o}C for 1 min and 72 ^{o}C for 3 min and a final prolongation at 72 ^{o}C for 5 min.

2.1.2 Cloning of the 6.5 kb Bam HI and the 9 kb Sac I fragments of MK5 gene

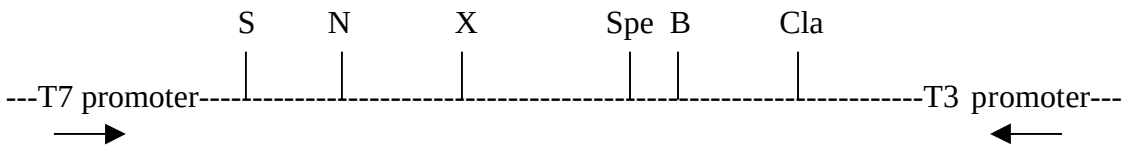
2 µg of MK5 genomic DNA was digested by Bam HI or Sac I for 2h at 37 ^{o}C. The digested DNAs were separated by 1% agarose gel. Then the two fragments, 6.5 kb Bam HI fragment and 9 kb Sac I fragment, were extracted from the gel.

Ligations were accomplished at 16 ^{o}C in 20 µl reaction system, consisting of 1×ligation buffer, 100 ng Bam HI fragment or 150 ng Sac I fragment, 40 ng cloning vector pBS II KS(+) digested by Bam HI or Sac I respectively, and 1 unit of T4 DNA ligase (GibcoBRL), Then 1 µl of ligation mixture was used for transformation with 50 µl of XL1 cell. Prior to inoculating, transformed bacteria were incubated at 37 ^{o}C for 1 h in 500 µl SOC (485 µl SOA +5 µl 1 M $MgCl_2$ + 5 µl 1 M $MgSO_4$ + 5 µl 2 M Glucose). 50 µl or the rest of transformation mixture plus 40 µl of 50 mg/ml X-gal and 30 µl of 100 mM IPTG was finally spread on 1.5%-agar LB plate containing 50 µg/ml ampicilin and incubated at 37 ^{o}C, overnight.

White colonies were picked up and the correct clones were determined by restriction analysis after Miniprep procedure. The plasmids from Maxiprep were kept at –20 ^{o}C.

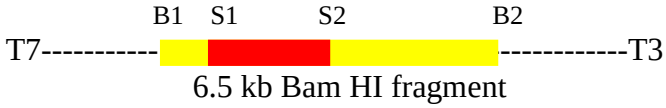
Scheme of pBluscript II KS(+):

B: Bam HI S: Sac I Spe: Spe I X:Xba I N:Not I Nh:Nhe I

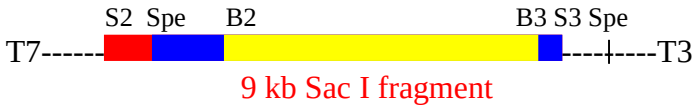


pBSIIKS(+): 3 kb

Clone I: pBS II KS(+)+Bam HI fragment



Clone II: pBS II KS(+)+Sac I fragment



2.1.3 Construction of the targeting vectors

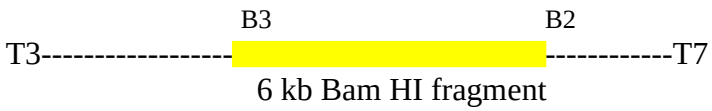
(1) Construction of the 1st targeting vector

The subcloning procedure was similar to the cloning procedure described above. Construction of the targeting vector included three main steps. After step (a), the clones could not be selected by X-gal and IPTG.

(a) Construction of Clone-B6k
Subcloning 6 kb-Bam HI-fragment from Clone II into pBS II KS(+)

4 µg of Clone II was digested by Bam HI for 2 h at 37 °C, then the digested DNA was separated by 1% gel. The 6 kb Bam HI fragment was extracted from the gel and was inserted into the same sites (Bam HI sites) of a new pBS II KS(+) vector (Clone-B6k).

Clone II

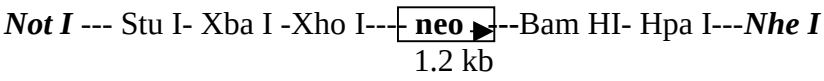


Clone-B6k

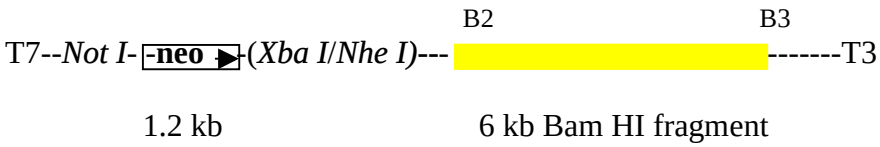
(b) Construction of Clone-NEO-B6k
inserting the "Not I-neo-Nhe I" box into the Clone-B6k by Not I / Xba I site

The neomycin-resistance gene (neomycinphosphotransferase) was got from ptV-O which contained thymidine kinase (TK) promoter.

1.2 µg ptV-O vector was digested by Not I and Nhe I for 2 h at 37 °C, then the 1.2 kb "neo" box fragment was extracted from the gel.



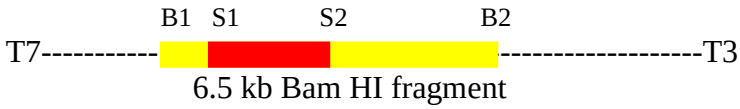
Clone-B6k was digested by Not I and Xba I for 2 h at 37 °C. To get Clone-NEO-B6k, 100 ng of the "Not I-neo-Nhe I" box fragment was inserted into 50 ng Clone-B6k, linearized by Not I and Xba I (compatible end with Nhe I).



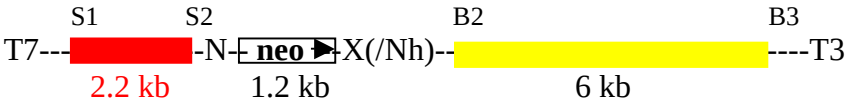
Clone-NEO-B6k

(c) Construction of the 1st targeting vector (1st TV).
inserting the 2.2 kb Sac I fragment of Clone I into Clone-NEO-B6k through Sac I sites

2 µg of Clone I was digested by Sac I for 2 h at 37 °C. To get the 1st targeting vector, the 2.2 kb Sac I fragment was extracted from the gel and inserted into the Clone-NEO-B6k, which was linearized by Sac I.



Clone I



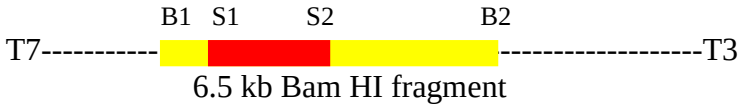
1st TV

As described above, the 1st TV has a neo-box (1.2 kb) which was inserted between two arms, a short arm (2.2 kb Sac I-F) and a long arm (6 kb Bam HI-F).

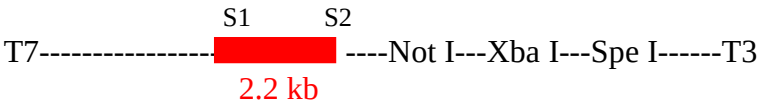
(2) Construction of the 2nd targeting vector

(a) Construction of Clone-S2,2k
Subcloning 2.2 kb-Sac I-fragment from Clone I into pBS II KS(+)

2 µg of Clone I was digested by Sac I for 2 h at 37 °C. The digested DNA was separated by 1% gel. The 2.2 kb Sac I fragment was extracted from the gel and was inserted into the Sac I sites of a pBS II KS (+) vector (Clone-S2,2k).



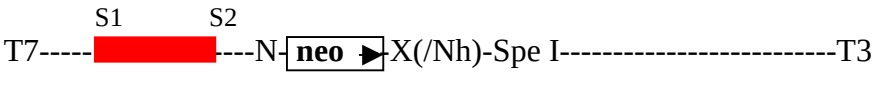
Clone I



Clone-S2,2k

(b) Construction of Clone-S2.2k-NEO
inserting the "Not I-neo-Nhe I" box into the Clone S2.2k by Not I site and Xba I site

Clone-S2.2k was digested by Not I and Xba I for 2 h at 37 ^{o}C. To get Clone-S2.2k-NEO, 100 ng of the "Not I-neo-Nhe I" box fragment (the same fragment as used in the 1st TV) was inserted into 50 ng Clone-S2.2k, linearized by Not I and Xba I.

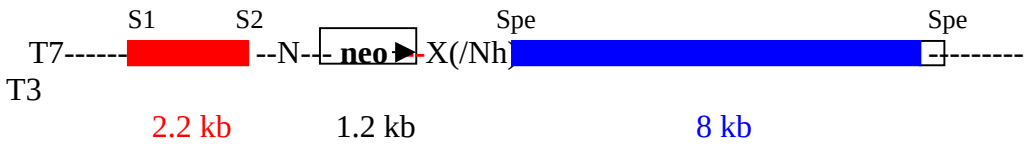


Clone-S2.2k-NEO

(c) Construction of the 2nd targeting vector (2nd TV)
inserting the 8 kb Spe I fragment of Clone II into Clone-S2.2k-NEO through Spe I sites

2 µg of Clone II was digested by Spe I, for 2h at 37 ^{o}C, and then the digested DNA was separated by 1% gel. To get the 2nd TV, the 8 kb Spe I fragment was extracted from the gel and was inserted into the Clone-S2.2k-NEO, linearized by Spe I.

Clone II



2nd TV

As described above, the 2nd TV has a neo-box (1.2 kb) which was inserted between two arms, a short arm (2.2 kb Sac I-F) and a long arm (8 kb Spe I-F).

2.1.4 DNA analysis

DNA was isolated by plasmid Minipreparations (Sambrook, Fritsch and Maniatis: Molecular Cloning, second edition, small-scale preparations of plasmid DNA, Section 1.25) and plasmid Maxipreparations according to the instruction of Plasmid Maxi Kit (QIAGEN). DNA was analysed by restriction analysis, sequencing and Southern Blot hybridisation.

(1) Sequence

7.5 µg DNA from Maxipreparation was sequenced with primer T 3, T 7 or custom primers in the "DNA-Service-Labor, Biozentrum der Martin-Luther Universität". The 2.85 kb region of MK5 genomic DNA between the first Sac I site to Spe I site (S1-Spe) has been sequenced and was named as MK5 relevant sequence (MK5-RS).

(2) Southern Blot Hybridization

3 to 5 µg DNA from Maxipreparation was digested with Bam HI or other restriction enzymes, and subjected to Southern blot. After pre-hybridization with Rapid-hybr. buffer for two hours, the membrane was hybridized with radioactive labelled probe (see Table 2-5) in the same hybridization buffer at 65 ^{o}C, overnight. Then the membrane was washed in 2 × SSC, 0.1% SDS at 65 ^{o}C for 30 min and 0.2 × SSC, 0.1% SDS at 65 ^{o}C for 30 min. After 4 to 24 hours exposure, the imager plate was scanned on the Phosphoimager machine (FUJIFILM BAS 1500).

2.2 Generation of MK5 knockout mice

2.2.1 Culture of ES cells

E14-1 ES (embryonic stem) cells which were derived from the 129/Ola substrain were the generous gift from Prof. Dr. Carmen Birchmeier. The feeder cells were neo-resistant MEFs (mouse embryonic fibroblasts) harvested from embryos at day E 13 by our own group.

(1) Culture of MEFs

MEF media

DMEM	500 ml
FCS	80 ml
NEAA	6 ml
pen/strep	6 ml

The MEFs were cultured at 37 ^{o}C in a 10% CO_2, 95% humidified CO_2 incubator.

(2) Inactivation of MEFs

Passage 4 of neo-resistant MEFs were growth arrested by mitomycin C treatment to serve as feeder. Mitomycin C was added to the media and final concentration of mitomycin C was 10 µg/ml. MEFs were treated with mitomycin C for 2 h at 37 ^{o}C, then washed with PBS for three times. Once the MEFs were inactive they could be stored in the incubator for up to 5 days before using as feeder.

(3) Culture of ES cells

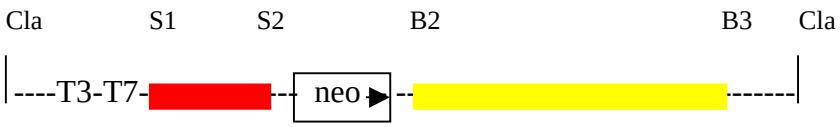
ES media

DMEM	500 ml
FBS	80 ml
NEAA	6 ml
Pen/strep	6 ml
Lif (ESGRO)	0.6 ml
50 mM ß-ME	1.2 ml

ES cells were grown on feeder layer in ES media at 37 ^{o}C in a 10% CO_2 , 95% humidified CO_2 incubator. Passage 3 of ES cells were used for electroporation.

2.2.2 Preparation of the linearized targeting vector

150 µg Targeting vector (TV) was digested with 125 unit of Cla I in a 1.2 ml reaction volume, at 37 ^{o}C for 2 h. Then the linearized TV was precipitated with ethanol and redissolved in 80 µl water.



1st TV

2nd TV

2.2.3 Electroporation and Selection of the ES cells

(1) Trypsinizing the ES cells

10 cm-dish ES cells were washed with 10 ml PBS for three times and then were trypsinized with 0.5 ml Trypsin/EDTA and PBS mixture (T/E:PBS=1:1), the dish was put in the incubator at 37 ^{o}C for 3 min. Then the ES cells were gently disassociated with a 1 ml pipette by carefully pipetting. The cells were checked under a microscope to verify that single cell suspension was formed.

ES cells were washed with PBS and harvested in a new tube. The cell pellet was resuspended in 1 ml PBS. 25µg linearized targeting vector was added into half of the 10 cm-dish ES cells (in 0.5 ml volume). The cell suspension with the linearized TV was kept in the hood for 10 min at room temperature. Half of 10 cm-dish ES cells were spread on four 15 cm-dishes feeder after electroporation.

(2) Electroporation

The ES cell suspension with linearized targeting vector was loaded into a 0.5 ml cuvette. Electroporation was carried out using a Bio-Rad Gene Pulser II at a capacitance of 500 microfarads, and at 240 V/cm voltage. After electroporation, the cells were kept on ice for 30 min and then were plated equally on the prepared four 15 cm-dishes feeder and were kept in the incubator.

(3) Selection of the ES cells

The electroporated cells were cultured under the same conditions as normal ES cells (2.2.1-(1)). G-418 was added to the culture media 24 h to 36 h after electroporation. Clones were selected for resistance to 385 µg/ml G-418. The media was changed daily for the next 5 to 7 days, 20 ml of fresh ES selection media /15 cm-dish/day.

2.2.4 Picking the clones

The clones were picked after 7 to 8 days after electroporation.

(1) 96-well plates were prepared with feeder one day before picking clones. One 15 cm-dish MEF feeder cells would provide for four 96-well plates.
(2) A microscope was placed into the hood and the UV light was turned on for at least 30 min.
(3) A 96-well plate was prepared with T/E, 50 µl /well.
(4) The media of the 15 cm-dish with ES clones was aspirated and the cells were washed twice with PBS. 20 ml fresh DMEM without FBS and other regents was added into the dish.
(5) The lid of the dish has been removed and the clones were picked with a 50 µl pipette under the microscope.
(6) Each individual clone has been picked and was placed into the individual wells with 50 µl T/E for about 20 min.
(7) The clone was disaggregated by carefully pipetting and was transferred to one well of a 96-well plate that already covered feeder layer and 150 µl ES media. 6 h later, the media was aspirated and each well was feeded with 200 µl fresh ES selection media.
(8) The cells were cultured in the presence of selection media under the same conditions as normal ES cells, the media was changed everyday.

2.2.5 Freezing ES clones

The ES cells were cultured in 96-well plate for about 5 to 8 days (depending on how large the clone was), then the cell clusters were dissociated with T/E+PBS and the cell suspension was divided into halves. One half was frozen and the other half was plated on gelatin-coated 48-well plate. The latter cells were allowed to grow (5 to 7 days) to confluence and were used as the initial material for the DNA extraction procedure.

(1) Preparation of gelatin substratum plate: 0.5 ml of 0.1% autoclaved gelatin was placed on each well of a 48-well plate. Having stood in the 37 ^{o}C incubator for more than 2 h, the gelatin was aspirated and the plate was dried in the hood.

(2) Preparation of the 25% DMSO freezing media for ES cells:

DMSO: FBS (for ES cells)=1:3

(3) Freezing ES cells: The ES media was aspirated from the well and the ES cells were washed with PBS for three times. 60µl T/E and PBS mixture (T/E:PBS=1:1) was placed into each well. Then the plate was put into a 37 ^{o}C incubator for 3 min. The ES cells were disassociated by carefully pipetting and then 30µl of cell suspension (half of the well) was transferred into a freezing cuvette which contained 30 µl ES freezing media (finally the ES cells were in 12.5% DMSO solution) and the ES cells were frozen slowly.

(4) The other half of ES cells (30µl) was cultured on gelatin-coated 48-well plate for 5 to 7 days.

2.2.6 Screening of the clones

(1) Isolation of genomic DNA from ES cells:

The other half of ES cells were cultured on gelatin-coated 48-well plate for 5 to 7 days, then the cell lysis was carried out as follows: the cells were washed twice with PBS, and 300 µl of lysis buffer was added per well.

The lysis buffer: 10 mM NaCl
10 mM Tris.Cl (pH 8.0)
25 mM EDTA
0.5% SDS
1 mg/ml Proteinase K (added freshly)

The plates were incubated overnight at 55 ^{o}C with lightly shaking, then 300 µl of phenol/chloroform was added (1 × vol). The plates were centrifuged at full speed at room temperature for 2 min and the upper phase was transferred to a new tube. 150 µl 7.5 M ammonium acetate (0.5 × vol) and 600 µl 100% cold ethanol (2 × vol) were added. After centrifugation at full speed for 10 min, the supernatant was discarded by inversion of the plates and the precipitated was washed twice with 70 % ethanol. The plates were left tilted to air dry for 30 min. The DNA was redissolved in 30 µl TE buffer.

(2) Screening the clones by Southern Blot Hybridization (see 2.1.4 (2))

25 µl of genomic DNA from step (1) was digested by Bam HI, and subjected to Southern blot. The membrane was hybridized with radioactive labelled probe. The experimental procedure was similar to the method of Southern Blot Hybridization above (see 2.1.4 (2))

2.2.7 Preparation of positive clones for microinjection

Rescue the positive clones: The positive clones were thawed very quickly in the 37 ^{o}C water bath and the cells were rescued in a 48-well plate with feeder layer. The 60 µl cell suspension was transferred to one 48-well with 500 µl ES media. 6 h later, the first

media was changed. ES cells were kept in a healthy, rapidly dividing state and the ES media was changed everyday. The passage 4 to 6 of healthy ES cells were frozen.

Microinjection was done with passage 5 or 6 healthy ES cells. The targeted ES cells were injected into C57BL/6J blastocyts by EMBL-Heidelberg Transgenic Service.

2.2.8 Generation of MK5 knockout mice

(1) The male chimeric mouse was bred with femal chimeric mouse or with female C57BL mouse to get brown mice (F1).
(2) The brown mice were genotyped by PCR and SB.
The brown MK5 +/- mice were bred to get knockout mice (MK5-/-) (F2).

2.2.9 Establishment of MK5 deficient embryonic fibroblast cell line (MK5-/-MEF)

(1) Preparation of mouse embryonic fibroblast cells from MK5-deficient mice

MEF cells were prepared from five E13.5 embryos obtained by intercrossing MK5 ko homozygous (-/-) mice. The brain, heart and other organs had been removed and the embryos were cut into small pieces and were treated with 0,5% Trypsin for 30 min. The MEFs had been harvested and finally plated on 15-cm culture plates. The MEFs were grown in DMEM medium containing 10% FBS and 1% pen/strep at 37°C in a humidified atmosphere of 5% CO_2.

(2) co-transfection of pSV40 and pREP8 vectors to the MEFs

After few passages, the cells were transfected with pSV40 and pREP8 vectors using Lipofectamin transfection Kit (LipofectAMINE PLUS, GibcoBRL). After 48 hours of transfection, histidinol was added to the medium (final concentration was 3 mM) to select the transfected cells.

Five days later, some clones could be seen. The clones were picked into 24-well plate and were cultured with histidinol for two more days. The cells were grown and expanded under the conditions above. Then the immortalized MK5 ko MEF cell line has been established.

2.3 Genotype of the mice

2.3.1 Isolation of genomic DNA from mouse tails

(1) 4-5 mm of mouse tail has been cut and was put into 250 µl extraction buffer.

extraction buffer : 100 mM NaCl
50 mM Tris.Cl (pH 8.0)
25 mM EDTA
1% Triton X-100

(2) 50µl of Proteinase K (1 mg) was added and the tail was incubated in the 300µl solutions at 55 ^{o}C overnight.

100 mg dry Proteinase K (Sigma, 2308)
4.75 ml H_2O
0.25 ml 1 M Tris pH 8.0
5 µl $CaCl_2$
(final concentration 20 mg / ml, 1 mg/50 µl, store at 4 ^oC)

(3) 300 µl (1 × vol) of Tris pH 8.0 equilibrated phenol was added. The solution was mixed vigorously and the centrifugation was performed at full speed at room temperature for 2 min.
(4) The aqueous phase was transferred to a fresh tube, an equal volume of chloroform/isoamyl alcohol (49:1) was added to the aqueous phase. After centrifugation at room temperature for 2 min. The aqueous phase was removed to a fresh tube.
(5) 150 µl (0.5 × Vol) 7.5 M ammonium acetate (NH_4OAC) and 375 µl (1.5 × Vol) isopropanol were added. The DNA was precipitated by centrifugation at room temperature for 5 min. The pellet was washed once with 70 % ethanol.
(6) After air-dry, the pellet was redissolved in 30 µl TE buffer and was kept at –80 ^oC.

2.3.2 Genotype mice by PCR

0.8 µl of genomic DNA (ca. 200 ng) from mouse tail was used as a template to perform PCR in a 25 µl reaction volume (HotStarTaq Kit, QIAGEN): 1 × PCR buffer, 0.2 mM each dNTP, 0.2 µM Primer MK5-RS-1833d (5' primer) and Primer MK5-RS-2803rc (3' primer), 1 unit of HotStarTaq polymerase with Biometra TRIO-Themroblock (Biotron, Germany).

The PCR programme: first denaturation and hot start at 96 ^oC for 15 min, 35 cycles of 96 ^oC for 1 min, 60 ^oC for 1 min and 72 ^oC for 2 min and 30 sec and a final prolongation at 72 ^oC for 5 min.

2.3.3 Genotype mice by Southern Blot

25 µl of genomic DNA from mouse tail was digested by Bam HI and subjected to Southern blot. The method was the same as above (see 2.1.4 (2))

2.4 Characterization of the mouse

2.4.1 Isolation of total RNA from tissues or from cells

(1) Isolation of total RNA from mouse liver, heart or spleen:

RNA isolation was based on the manual of RNeasy mini kit (QIAGEN). 150 to 200 mg tissue was homogenized in 1600 µl RLT solution. After centrifugation at full speed at room temperature for 3 min, the supernatant was transferred to a new tube. One volume of 70% ethanol was added and mixed well. Mini columns were applied to 2-ml collection tubes, about 700 µl volume was added to the mini column each time. After centrifugation at full speed at room temperature for 1 min, the column was washed with 700 µl RW1 solution by centrifugation as above. The column was washed with 500 µl RPE solution by centrifugation at full speed at room temperature for 1 min for three times. Then the column was dried by centrifuging at full speed at room temperature for 2 min. 50 µl of DEPC water was added to the column and the RNA was harvested by

centrifugation at full speed at room temperature for 1 min. The concentration of RNA was measured and RNA was kept at –80 °C.

(2) Isolation of total RNA from macrophages:

The total RNA of macrophages was isolated by using peqGold RNA Pure kit (peqLab). 5×10^6 cells were harvested in a tube, 1 ml peqGOLD RNAPure™ solution was added, mixed well by pipetting. The mixed solution was kept at room temperature for 5 min, then 200 µl chloroform was added. After vortex for 15 seconds, the solution was kept at room temperature for another 10 min. After centrifugation at full speed at room temperature for 5 min, the upper phase was transferred to a new tube. 500 µl isopropanol was added and the solution was kept at room temperature for 15 min. The RNA was precipitated by centrifuging at full speed at 4 °C for 10 min. The RNA pellet was washed with 70% ethanol. After air-dry for 30 min, the RNA was re-dissolved in 50 µl DEPC water. RNA concentration was measured and RNA was kept at –80 °C.

(3) RT-PCR

Reverse transcription (RT) was performed in a 20 µl consisting of 1 × display THERMO-RT buffer, 0.5 mM each dNTP, 0.8 µg total RNA, 1 µM T25 primer, 2 µl display THERMO-RT terminator mix. The reaction was carried out at 42 °C for 40 min, stopped by at 65 °C for 10 min. The RT-solution was kept on ice for PCR or kept at –80 °C until used.

PCR was carried out in 25 µl containing 1 × PCR buffer, 0.2 mM each dNTP, 0.2 µM each primer, 1 µl RT-solution and 2 units of HotStarTag polymerase (QIAGEN) with thermal-cycles including the first 96 °C for 15 min, then 35 cycles of 96 °C 1min, 50 °C for 1min and 72 °C for 1 min, and a final prolongation at 72 °C for 5 min.

2.4.2 Western immunoblotting analysis

Proteins were extracted from the same amount of (0.2×10^5) cells and were resolved by electrophoresis in 7.5-20% gradient polyacrylamide-SDS gels. Then the proteins were transferred to nitrocellulose membranes (Amersham), incubated with blocking solution (5% non-fat milk in 10 mM TrisCl and 150 mM NaCl) for 1 h, then probed for 22 h with the 2 µg/ml of anti-PRAK (Upstate) in blocking solution at 4 °C. Washed with PBS then the blots were subsequently probed with the rabbit anti-sheep HRP-conjugated IgG (1:2000 dilution was used). The membranes were washed with PBS 20 min for three times. Then the “Enhanced Chemiluminescence system” was used for detecting the secondary antibody. Blots were exposed to the film (Hyperfilm ECL).

2.4.3 MK5 Kinase Assay

(1) solutions for MK5 kinase assay

Lysis Buffer: 20 mM Tris-acetate (pH 7.0)
0.27 M Sucrose
1 mM EDTA
1 mM EGTA
1 mM Orthovanadate
10 mM β-glycerophosphate

50 mM Sodium Fluoride
5 mM Sodium Pyrophosphate
1% triton X-100
0.1% (v/v) b-Mercaptoethanol (add freshly)
Protease inhibitor cocktail (Roche, 1 tabet for 10 ml buffer, add freshly)

Substrate Buffer: 0.5 mM EGTA
0.5 mg/ml BSA
30 µM PRAK substrate (or 5-10 µg Hsp25 for 50 µl volume)
50 mM Tris-HCl (pH 7.5)

Hot Buffer: 75 mM $MgCl_2$
0.5 mM cold ATP
2 µl [γ-^{33}P]-ATP (fresh)

(2) protein extraction

Cells were washed with PBS, then lysed in 500 µl lysis buffer, after vortex for 10 seconds, the lysate was put on ice for 5 min. After centrifugation at full speed at 4 ^{o}C for 10 min, the supernatant was transferred to a new tube.

About 100 mg tissues were homogenized in 500 µl lysis buffer. The solution was put on ice for 10 min. After centrifugation at full speed at 4 ^{o}C for 10 min, the supernatant was transferred to a new tube.

The protein concentrations were determined with the "Bio Rad Protein Determination Kit". 400 µg (for Hela cells) or 1000 µg (for MEFs) proteins were used per kinase assay.

(3) immunoprecipitation of MK5

Kinase was immunoprecipitated from the lysate by using 2 µg PRAK antibody (Upstate) for 400 to 1000 µg proteins. After 1 h mixing at 4 ^{o}C, 30 µl pre-equilibred (in lysis-buffer) protein G-agarose (50% slurry) was added and mixed for another hour.

The precipitate was washed 3 times with lysis buffer containing 0.5 M NaCl, two times wash with 50 mM Tris-HCl pH 7.5.

(4) kinase assay

40 µl substrate buffer was added per assay. After preheating at 30 ^{o}C for 3 min, 10 µl hot buffer was added per assay. Kinase assay was performed at 30 ^{o}C for 20 min in 50 µl volume.

20 µl aliquot was spotted on Whatman p81 paper and washed extensively in 1% phosphoric acid before counting

2.4.4 Induction of endotoxic shock

Administration of LPS and D-Galactosamine: LPS and D-Galactosamine were purchased both from Sigma. The mice were challenged with intraperitoneal (i.p.) administration of 1 μg of LPS and 20 mg of D-galactosamine or 10 μg of LPS and 20 mg of D-galactosamine. After administration, the experimental mice were observed for 24 h and the number and time of sacrificed mice were recorded.

2.4.5 Immunization

Sex-matched animals 6-12 weeks old were immunized by intraperitoneal administration of 100 μg of human serum albumin coupled to DNP in incomplete Freunds adjuvant (IFA). Serum samples were collected from the mice 10 days after primary immunization or secondary immunization. Levels of DNP-specific immunoglobulin (Ig) isotype were determined by ELISA using DNP-Albumin as a capture agent and goat anti-mouse isotype-specific antibodies directly conjugated to alkaline-phosphatase (Southern Biotechnology Associates). Anti-mouse Ig capture antibodies were used for the measurement of non-specific antibodies level.

After immunization for 12 days, the mice were killed and the germinal centres in the spleen were examined by histology. The centrocytes were stained by rhodamine-labelled peanut agglutinin (PNA) and the B-cells were co-stained by FITC-B220-pan-B.

2.4.6 Measurement of cytokines production

The cytokines were determined with an ELISA kit (Quantikine^{TM}, R&D Systems) according to the manufacturer's instructions by Chrite (in Berlin).

2.4.7 Statistical analysis

Differences between groups were analysed by t-test (SigmaPlot 4.0). A P value <0.05 was considered significant.

III. RESULTS AND DISCUSSIONS

1. Analysis of MK 5 Gene and Construction of Targeting Vector

MAPK cascades transduce the signals from cell membrane to the nucleus and play important roles on cell survival and adaptation. To learn more about MAPK functions, it is very important to investigate the normal and pathophysiological functions in the whole organism. In recent years, with the analysis of the mutant mouse strains produced by gene targeting, we have gained new understanding about the functions of MAPK. Some deficient mice on MAPK signal cascades have been produced. In p38 MAPK cascade, MKK3 (Lu et al., 1999), p38α (Adams et al., 2000; Tamura et al., 2000), MK2 (Kotlyarov, 1999), ATF-2 (Reimold et al., 1996), partial and complete of CREB (Bourtchuladze et al., 1994; Rudolph et al., 1998) and MSK1/2 (Wiggin et al., 2002) deficient mice have been generated and analysed.

MK5 or PRAK (p38-regulated/activated kinase) was identified as serine/threonine kinase phosphorylated by p38 independently by two groups in mouse and human leading to the two different names (Ni et al., 1998; New et al., 1998). Meanwhile, it is highly probable that PRAK is the human homologues of murine MK5.

Both MK2 and MK5 are direct downstream substrates of p38 MAPKs. MK5 displays 45% amino acid identity to MK2 (Ni et al., 1998). The phylogenetic tree (Fig. 3-1) shows the phylogeny among six MAPKAPKs and the distances are related to the degree of divergence between the sequences (Corpet, 1988). From this phylogenetic tree we can see that the phylogenetic conservation between MK2 and MK3 is higher than that between MK2 and MK5. This indicated that MK2 and MK5 may share diverse functions, and their overlap functions are not as much as that between MK2 and MK3. Since we already produced MK2 ko mice (Kotlyarov et al., 1999), therefore, to mutate MK5 gene would be more important than to mutate MK3 gene by gene targeting. And to produce MK5 ko mice would also help us to understand p38 MAPKs pathway more clearly.

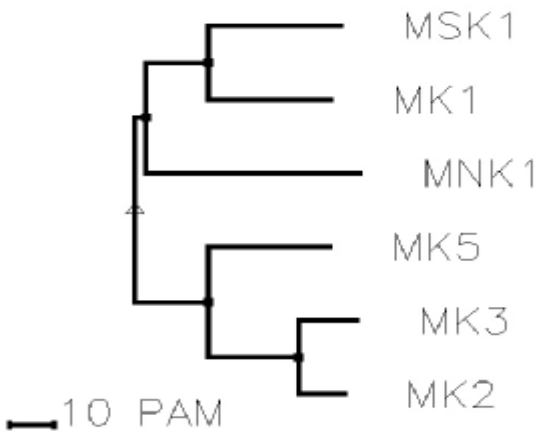


Fig. 3-1: Phylogenetic tree of six MAPKAPKs.
The phylogeny of six MAPKAPKs (MSK1, MK1, MNK1, MK5, MK3 and MK2) are showed in the figure. The phylogenetic conservation between sequences are related to the degree of divergence of the sequence. Δ: tree root; PAM: distance between sequences

MK5 genomic DNA was isolated from a P1 clone obtained from “Genome Systems”. Firstly, we examined the MK5 genomic DNA in P1 and ES cell DNA by PCR with primers, MK5-1d, MK5-1 rc, MK5-2d and MK5-2 rc, which were designed according to the MK5 cDNA (AF039840). Now we know that the primers are located in exon 7 to 10 of MK5 gene: MK5-1d in exon 7; MK5-2d in exon 8; MK5-1 rc in exon 9 and MK5-2 rc in exon 10 (Fig. 3-5; Table 3-1). With different combinations of these primers, we obtained the same length PCR products both from ES cell DNA and from MK5 genomic DNA (Fig. 3-2; Table 3-1 and Table 3-2). This indicated that the genomic DNA contained MK5 cDNA sequence.

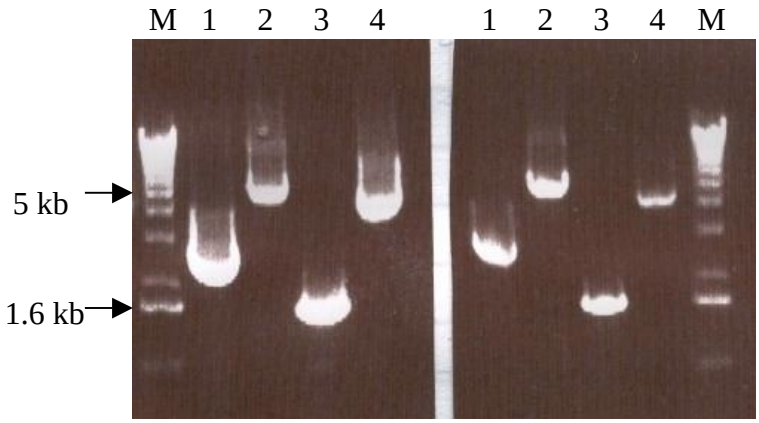


Fig. 3-2: Analysis of MK5 genomic DNA and ES cell DNA by PCR.
(a): PCR was performed using MK5 genomic DNA as template, with the primer composition of No. 1 (MK5-1d and MK5-1rc), No. 2 (MK5-1d and MK5-2rc), No.3 (MK5-2d and MK5-1rc) and No.4 (MK5-2d and MK5-2rc); 2.5 kb, 5.5 kb, 1.7 kb and 4.5 kb PCR products were obtained respectively (indicated in Table 3-2). (b): PCR was performed using ES cell DNA as template with the same primer composition of No. 1 to No.4, the same length PCR products as in (a) were obtained. M: marker

Table 3-1: Location of primer

Primer	Location (exon)	Description
		MK5 cDNA (AF039840)
MK5-1d	7	MK5 cDNA 1228-1252, 5’ primer
MK5-2d	8	MK5 cDNA 1315-1341, 5’ primer
MK5-1rc	9	MK5 cDNA 1522-1496, 3’ primer
MK5-2rc	10	MK5 cDNA 1612-1589, 3’ primer

Table 3-2: Results of PCR analysis of MK5 genomic DNA and ES cell DNA

No.	Primer Composition	PCR product
1	MK5-1d+MK5-1rc	2.5 kb
2	MK5-1d+MK5-2rc	5.5 kb
3	MK5-2d+MK5-1rc	1.7 kb
4	MK5-2d+MK5-2rc	4.5 kb

By further analysis of the genomic DNA, we found that the P1 clone contains a 12.5 kb MK5 genomic DNA. In this region, we could find at least five exons and some restriction sites (Fig. 3-3). Very recently, we obtained a 163 kb MK5 genomic DNA sequence from "Celera Genomics". Analysing the sequence of this 163 kb genomic DNA, we found that MK5 cDNA (AF039840) was mapped in a 15 kb region. This 15.5 kb region contains exon 2 to exon 14 of MK5 gene (MK5 cDNA 743-2128, the sequence was not exact enough to detect the first exon of MK5 cDNA 707 to 742) and some restriction sites. The 15.5 kb MK5 genomic DNA was shown in Fig. 3-5.

The 2.85 kb fragment between Sac I and Spe I (S1 to Spe, Fig 3-3) of P1 clone, has been sequenced, named MK5 relevant sequence (MK5-RS, see Fig. 3-4) which contains two exons of MK5 gene. MK5-RS 665 to 773 is identical with MK5 cDNA 991 to 1099 which encodes catalytic subdomain IV of MK5; MK5-RS 1943 to 2032 corresponds to MK5 cDNA 1100 to 1189 (90 bp) which encodes catalytic subdomains VI a and VI b of MK5 (Fig. 3-4). Comparing the 15.5 kb MK5 genomic DNA structure to our 12.5 kb MK5 genomic DNA (Fig. 3-3), we obtained the same structure and organisation of the gene. Alignment of our 2.85 kb relevant sequence of MK5 with the 15.5 kb MK5 genomic DNA, we found that a 356 bp fragment in intron 4 was missed in our 2.85 kb fragment (data not shown), this difference in sequences may be due to the difference in mouse substrains used (E14-1 ES cells derived from 129/Ola mice).

Two targeting vectors were constructed according to the structure of 12.5 kb MK5 genomic DNA (Fig. 3-3). Before starting the electroporation, we checked MK5 genomic DNA by Southern blot analysis with probe P2 and it was shown that MK5 gene was not a multicopy gene (Fig. 3-6) so it is possible to mutate MK5 gene by gene targeting.

In our 2nd TV, an internal 0.8 kb Sac I–Spe I (S2-Spe, Fig 3-3) MK5 genomic fragment was replaced by a neo cassette from ptV-O (Fig. 3-3). The deleted region encompasses one exon encoding amino acids 131–161 of MK5, which contains a subdomain of the catalytic region of MK5 required for protein kinase activity. With the 2nd TV, Southern blot analysis of 300 independent G418-resistant clones revealed two clones, clone 60 and clone 149, which resulted from the desired homologous recombination event (targeting frequency 0.6%) (Fig. 3-7). When hybridized with external probe P1 or the internal probe P2, clone 60 and clone 149 both not only had the wild type 6.5 kb signal, but also showed the 4.4 kb homologous recombination signal. Clones 56, 57 and 58 only had the wild type signal and were shown for comparison. When hybridized with the 1.2 kb neo probe P3, clone 60 and clone 149 only had one 4.4 kb signal, while clone 56 had a large fragment contained neo signal, clone 57 had four different signals which all are larger than 4.4 kb and clone 58 had no neo signal. These results showed that the MK5 gene had been disrupted in clone 60 and clone 149 by homologous recombination and that only one homologous recombination happened in the two positive clones. No additional non-homologous integration events as seen for clones 56 and 57 could be detected in these clones.

Unfortunately, we screened more than 1600 electroporated ES cell clones with the 1st TV, but did not find any positive clones. The deleted region and the long arm were different between the two TVs. The long arm of the 2nd TV is 8 kb, longer than the 1st TV's (6 kb). The deleted region in the 2nd TV is 0.8 kb, shorter than the deleted region (3.3 kb (S2-B2, Fig 3-3)) in the 1st TV. That may be the reason for the higher recombination frequency obtained with the 2nd TV.

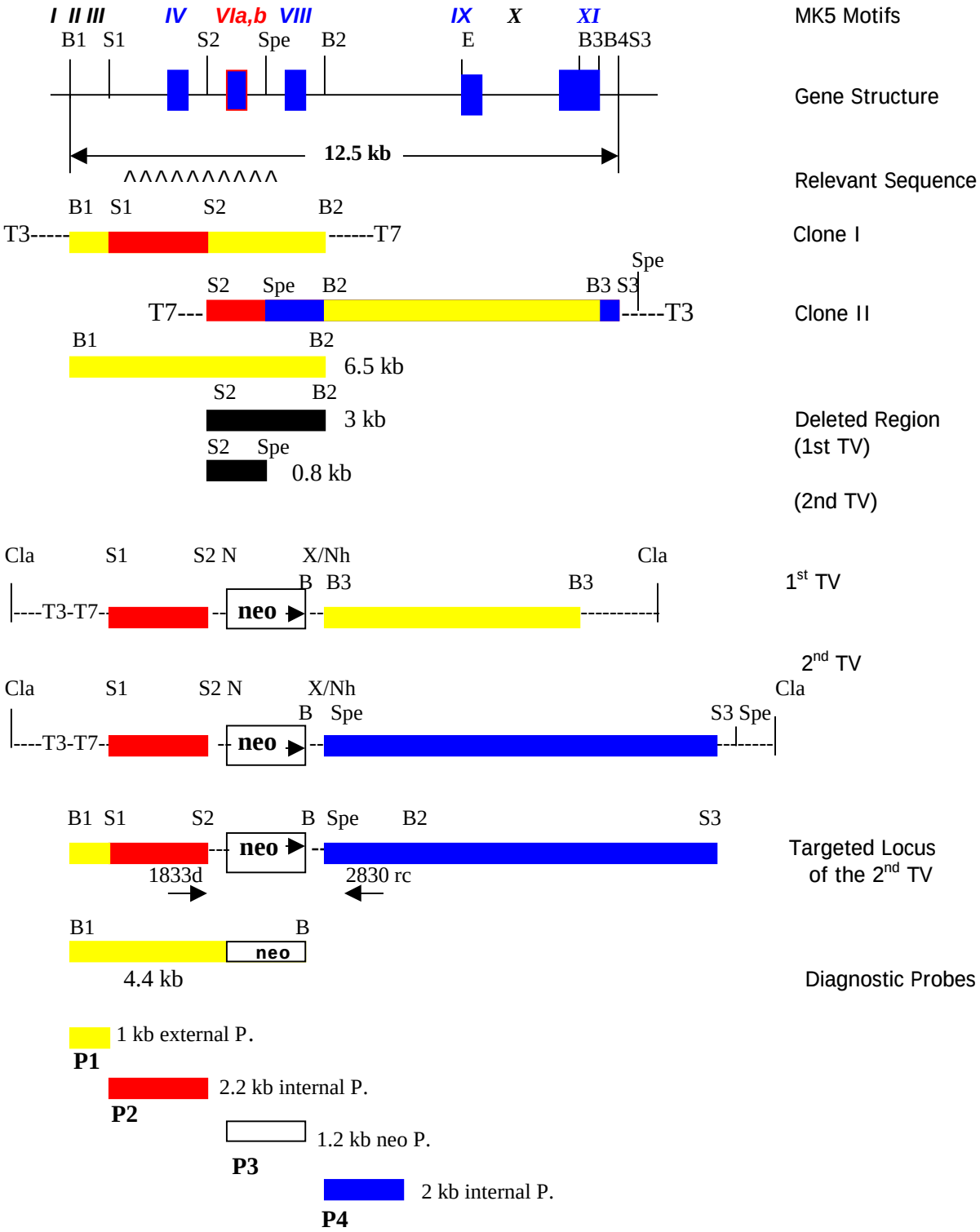


Fig. 3-3: Structure of MK5 gene and strategy applied to mutate the MK5 gene.
Structure and partial restriction map of MK5 gene, clone I (6.5 kb Bam HI fragment clone) and clone II (9 kb Sac I fragment clone), two targeting vectors, targeted locus of the 2nd TV and diagnostic probes. The MK5 sequence motifs encoded by different exons (shown by blue boxes) are indicated (roman numerals, catalytic subdomains). The neo cassette was inserted into the exon encoding subdomainVI; the mutant locus is expected to encode a truncated kinase that lacks amino acids 131 to 161 of MK5, which are essential for enzymatic activity. B: Bam HI; Cla: Cla I; E: Eco RV; Nh: Nhe I; N: Not I; S: Sac I; Spe: Spe I; X: Xba I. 1833d: MK5-RS-1833d, 5' primer; 2830rc: MK5-RS-2830rc, 3'primer; P: probe.

```
TCGTCAGCCC TGGGGMATCT TAHNCATTTG CTCCCTTCCT CCACCATTAT   50
TTTGATTACT TTCTCTTTTC AAAAAATGTT TAAGGGCTTG GGGATTATAG
ATTAGTGATG GAGTGCTTGC CAACCGTACC TAAGGCCCTG GATTTACTCT
CTAATGCCCA GTGTGTGTGT GCGTGTGTGT AGTTAAAAGG AAAATAAAAC        200
ATTAAAGAGC CAGGTATAGT TGGTGCATGC CTATAATCCT AGCCCTTGAG
AAGCTGAGGC AGTGTCTCAG GCTTCGGGAA GTGCGCATGG GAAAGGGGGT
ACAGAAGCTG TGCTGGTAAA GCCAGTGGGA AGTCGATTGG GGTTCCCAAG
TGTGCTCTGA TGCTGAAAGC ATTTGGATGC AAGCAGAAGC ATTCATAGTC        400
CTAGTGGGAC TAGGAGGCAG AAGTTCATGT TTGGTTTTTT CTTTTTCTTC
CTTTTTACAT TTTGGTGGAG AATATGAGAG TCCTTGCACT TAGTTAGTGG
CTAGCTTACC CCGGACACAG TGGCTGCATA CTTTGTAGAT GACATCACTT
TAGGTCAGTG TTTGATGCTT CAGGGTTTTC CTTTTCCTTT TTTCCTTTGT        600
TTTTTTCCTT TTCCTTTTTT TTCTTTTTTT TTTAAACTTA ACAAAATCAA
ATGCTTTCAA ACAGGGCTCG ACTCTTAATT GTAATGGAGA TGATGGAAGG
GGGAGAGCTA TTTCACAGAA TCAGCCAGCA CCGGCACTTT ACAGAGAAGC
MAGCCAGCCA AGTAACAAAG CAGGCAAGTT GACCCCGGGT ACCCGGCCAA        800
ACTGCGACTA CCTTGTTAAG CAGTACAATT GCATTGTGGG AAGGGAAGCT
TAAAGAAAAG CTCCATTTGA CCTCTCATTG CCTGCTTTGT TATAATCAGA
AAGGAAGGAC ATCTTCCTAA AAGTGCCACT GACCCTTTAT ACTTTGGGAG
GGAGGATTGG ATTTTTAATT GCTTGTCACC TTTCCCAAGG ATGCTCTGAG       1000
AGTTTCCCCC ACGGTCTTAA CTGTGGACAG CTGTGGGCAG GTAGCATTTG
ATGCTGTTGC TATCACCTGA AAGACCTTCA GGCATTTGTA CCTAAGATGT
TTCTTGCATA AATCACCCAT CATTTCTGTG GGTTTTTGTA ATAGTGAGTG
ATTCTTGGTT ATATTCTATT TTATGTCCCA TACAATTNCC CTCATAGAGG      1200
TGACAGGCCT GTCTTCATAC CACGCADGTA GACCATAGGA AAGCDTGANG
CCACAACAGT TAGCATTGTG AGAAAGCTCG TCAGATTGTC CACCCTCTAT
TCTCTAGTGT CTCTGGATGC CYCCTTTCTG ATTGACTAGG GTTAACCACT
ATGTGAGTGC TGAGAAGATC CACAAGAGTT NGANGAACTA TTTAACTTTT      1400
AAATTACACC ATTCATTTTT CAACATTAAG ACTAAAGGAT GAGATGTGAC
CATCTGTAAA ATAAAAGCTG TCTCAGTCTA CACAGACAGA TGCATTTTCG
TGAGATAGGA AGTCAAGCTG GCTGGATGGA AGGTATTGCC TGCCATTCAG
CTCCTTAGGG TGCAGGTATC TGGAGGAGCA GCCCTGGGGT CACAGCAGGG      1600
GTCCACTNGG AAGCCCTTCT TATTGTGGAC TGCTGCCTCT GGGTCCCCAC
AGTACCTGTC ACAGCACAGG TAAAGGAGTG GCCAGGCATC TTACAGGATG
TGTCCACCGC TTTATTCTGA CAGAGGATCA TTTGTAGTCG TGCTTGTTCA
GTAAATATAG GTTATAGTTA AACTAAGGCC AATGTATTTG TGTTTCTTGG      1800
TGTCCCAAGT CTTTCTTAGT TTCTCTCGTG TACGTAACAC TAGCCACAGT
TGTAACTGAC TTAAACCCCT GAGCTCTCAC ACTAGGGGTG TTAGAGATGG
TGCTTTTCTA AGAGGTGGCT GGTCCACTGT GCCCTTTTCC AGATAGCCCT
GGCTCTACAG CACTGTCACT TGCTAAACAT TGCGCACAGA GACCTCAAGC       2000
CTGAAAATCT GCTTTTCAAG GATAACTCTC TGGTGAGAAC AACTGCTTTT
CCTCATTTGA TGCTGTCACT TTGTTTTGTT TTGTTCAGTT TCTGTTTTTT
AAAGATAGAG TCTGTCTATC CCAGGCCAGC CTTGAACTTA TTCTGTAGCT
GCAGATGTCC TCGATCTTCT GACCTCCTGC CTCCACCTCC TTTTGCGTGT      2200
TGGGATTCCA GGTATGCATC AGCAGACTCA GTTTATGTGA CTGTGGGGTT
GAATCCAGGG CTTCGCACCC AGTAGGCCAG CACTGTGCCA ACTGATGTAC
ATCCTCAGGC TGGTTTAATT TTTGCCATAC TGTCTTGCTT TGTGGACCAG
GCTGGCCCGG AACCCATGTA AACGAGGTTG GCCTAAAACT CAGGAAGCCT      2400
TTGCTTCCTG AGTGCTGGGA TTGCAAGTGT GTGCTTCCTG CTGCTGTACT
GACCCCGTGC ACAGGAGGTT NAGGCATGTG CTTCTAATAC TTCAGCGCCC
ACTCTTTCCT TGTTAGGGCC TCAGAACTGT TGCCACACCC ATGGCATGGG
TGATTTGGAT TGTACAGATA CATCTGTGGG GCTTATGAAC TTGCATATTA      2600
TCTGGACAGC CTTTGGGGAG AGGCAGGAAT TGACATGCTG TTACATAGTT
CAGTTAGAGT TGCTGTTAGC CCTGATATAG GCTGTGTTCT TTTCCTGGTT
TCCTGTCCAT ATTCAGTATA CTAGTCAGTT GCTCCAGTTG CCAGTYTTCA
GAGTGAGGCT GGGTGGATGA GAGGAGGGCC AGCCTGTTAG TTAAGACTCT      2800
GTAACTCAGA GCTGTGCTTA CAAGTATATG TCTGGGGGCT TGTKAGATKG
```

Fig. 3-4: Relevant sequence of the MK5 gene.
MK5-RS 665 to 773 (blue)= MK5 cDNA 991 to 1099, exon 5 which encodes catalytic subdomain IV of MK5; MK5-RS 1943 to 2032 (red)=MK5 cDNA 1100 to 1189, exon 6 which encodes catalytic subdomains VI a and VI b of MK5.

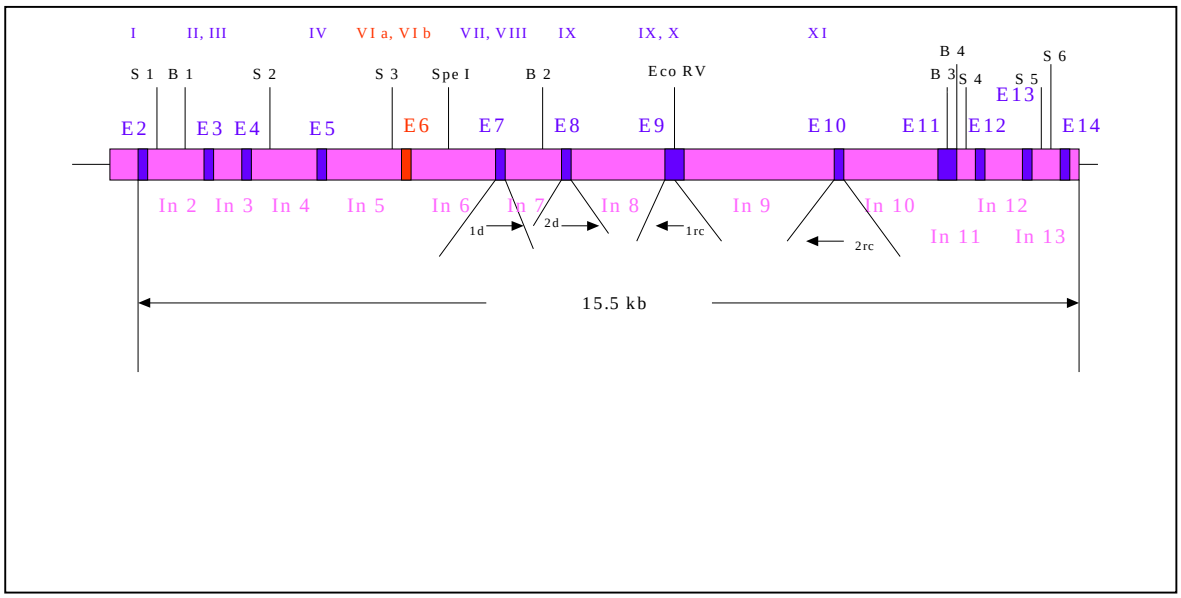


Fig. 3-5: Structure of the 15.5 kb MK5 genomic DNA.
Structure and partial restriction map of the 15.5 kb MK5 genomic DNA. The MK5 sequence motifs encoded by different exons (shown by blue boxes) are indicated (roman numerals, catalytic subdomains). I-XI: MK5 catalytic subdomians; E 2-14: exon 2-14; In 2-13: intron 2-13; S 1-6: 6 Sac I sites; B 1-4: 4 Bam HI sites; 1d: 5‘ primer, MK5 cDNA 1228-1252; 2d: 5‘ primer, MK5 cDNA 1315-1341; 1 rc: 3 ‘ primer, MK5 cDNA 1522-1496; 2 rc: 3‘ primer, MK5 cDNA 1612-1589.

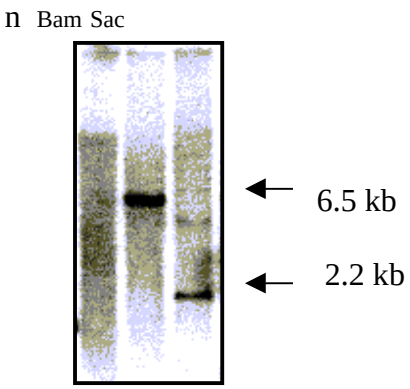


Fig. 3-6: Southern blot analysis of mouse genomic DNA with probe P2.
The non-digested mouse genomic DNA (n) and the mouse genomic DNA digested by Bam HI (Bam) or by Sac I (Sac) hybridized with 2.2 kb internal probe P2. No positive signal could be detected in non-digested genomic DNA; one band at 6.5 kb was detected in lane Bam, and one band at 2.2 kb was detected in lane Sac.
(The Bam HI fragment, Sac I fragment and probe P2 have been indicated in Fig. 3-3.)

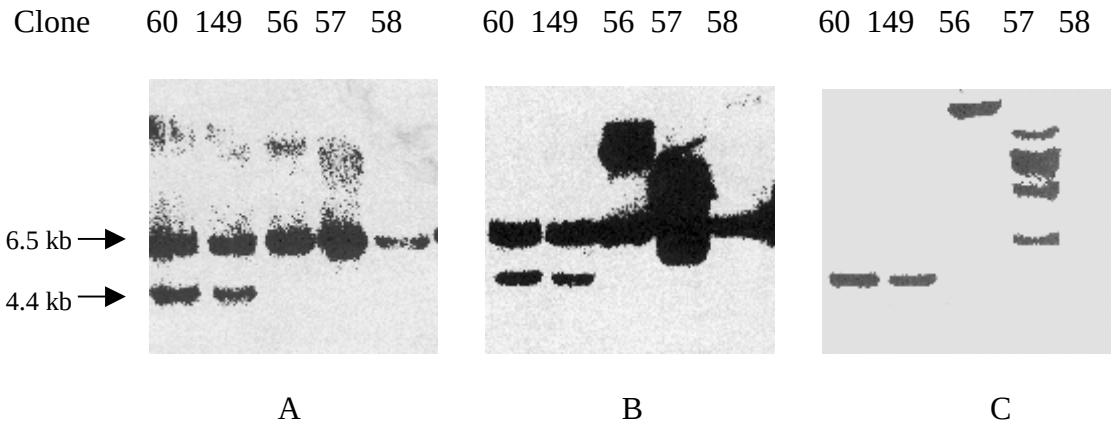


Fig. 3-7: Southern blot analyses of the electroporated ES cell clones.
ES cell DNAs were digested by Bam HI and then were hybridized with 1 kb external probe P1 (A), 2.2 kb internal probe P2 (B) and 1.2 kb neo probe P3 (C). For clone 60 and clone 149, two bands at 6.5 kb and 4.4 kb were detected in A and B, but only one band at 4.4 kb was detected in C. For clone 56, 57 and 58, one band at 6.5 kb was detected in A and B. One large band was detected in clone 56 in C; and four different bands which all are larger than 4.4 kb were detected in clone 57 in C; no signal was detected in clone 58 in C. (The Bam HI fragments and the probes have been indicated in Fig. 3-3.)

2. Generation and Characterisation of MK5-Deficient Mice

Chimeric mice were generated by injecting the targeted ES cells into C57BL blastocysts by EMBL-Heidelberg Transgenic Service. 20 chimeric mice have been obtained from the two positive clones (see Table 3-3). Both clone 60 and clone 149 resulted in germline transmission of the disrupted MK5 allele. Heterozygotes (+/-) were intercrossed to generate homozygous mutant mice (-/-) that were identified by PCR and Southern blot analysis of genomic DNA.

Table 3-3: 20 chimeric mice. M: male; F: female

Mouse number	Date of birth (day/month/year)	Sex	Strain	Chimeric percentage
520787	24.09.00	M	Clone 149	90%
520788	24.09.00	M	Clone 149	90%
520789	24.09.00	M	Clone 149	90%
520790	24.09.00	M	Clone 149	80%
520791	24.09.00	M	Clone 149	80%
520792	24.09.00	M	Clone 149	70%
520793	24.09.00	M	Clone 149	40%
520794	24.09.00	F	Clone 149	100%
520795	24.09.00	F	Clone 149	90%
520796	24.09.00	F	Clone 149	80%
520797	24.09.00	M	Clone 149	90%
520798	24.09.00	M	Clone 149	30%
520799	24.09.00	F	Clone 149	90%
520800	24.09.00	F	Clone 149	90%
520801	24.09.00	F	Clone 149	80%
520802	24.09.00	F	Clone 149	70%
520803	30.09.00	M	Clone 60	90%
520804	30.09.00	M	Clone 60	50%
520805	30.09.00	F	Clone 60	40%
520806	30.09.00	M	Clone 60	90%

The MK5 knockout mice were viable and fertile, grew to normal size and did not exhibit obvious behavioural defects. The genotypes of 53 offspring from MK5 heterozygous (+/-) mice intercrossing were examined and the mice were present at a frequency consistent with Mendelian inheritance (Table 3-4).

Table 3-4: Birth rate of the mice
(a): 53 offspring from 7 different parents.(b): ratio of the genotype. M: male; F: female

(a)

Parents' Cage No.	Cage No.	Mouse No.	Sex	Genotype	Genotype		
					++	+-	--
358	567	1406	M	+-	1	3	1
		1407	M	--			
		1408	M	++			
		1409	M	+-			
		1410	M	+-			
	568	1411	F	+-	1	3	1
		1412	F	+-			
		1413	F	--			
		1414	F	+-			
		1415	F	++			
381	571	1426	M	+-	1	2	0
		1427	M	+-			
		1428	M	++			
	572	1429	F	++	2	0	1
		1430	F	++			
		1431	F	--			
389	603	1518	F	+-	0	3	1
		1519	F	--			
		1520	F	+-			
		1521	F	+-			
	604	1522	M	+-	1	2	0
		1523	M	+-			
		1524	M	++			
382	598	1506	F	--	0	2	1
		1507	F	+-			
		1508	F	+-			
	599	1509	M	+-	1	2	0
		1510	M	+-			
		1511	M	++			
384	615	1561	M	+-	0	3	1
		1562	M	+-			
		1563	M	+-			
		1564	M	--			
	616	1565	F	--	1	2	1
		1566	F	+-			
		1567	F	+-			
		1568	F	++			
383	641	1651	M	++	1	0	1
		1652	M	--			
	642	1653	F	+-	0	1	2
		1654	F	--			
		1655	F	--			
397	643	1656	F	+-	2	3	1
		1657	F	+-			
		1658	F	++			
		1659	F	--			
		1660	F	++			
		1661	F	+-			
	644	1662	M	+-	2	3	0
		1663	M	++			
		1664	M	+-			
		1665	M	+-			
		1666	M	++			

(b)

Amount of Mice	Genotype		
	+/+	+/-	-/-
53	13	29	11
Ratio	1	2.2	0.8

(1) Screening and Genotyping of the Mice by PCR

Using primers MK5-RS-1833d and MK5-RS-2830rc, we screened mouse-tail DNA. The PCR product of wt DNA was about 0.97 kb and the product of homologous recombinated DNA was about 1.2 kb (Fig. 3-8). In Fig.3-8 (b), No. 13, 18 and 25 were wild type mice (+/+); No. 14, 15, 16, 19, 20 and 28 were MK5 heterozygous mice (+/-); No. 17, 21 and 34 were MK5 ko mice (-/-).

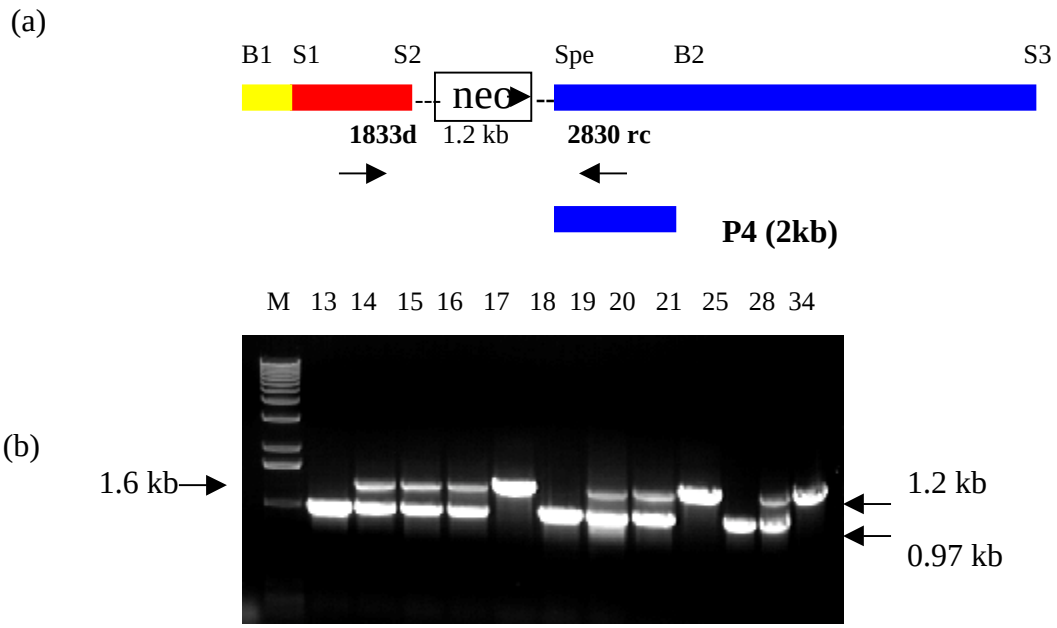


Fig. 3-8: Screening and genotyping mice by PCR.
(a): Structure of homologous recombination DNA, primers for PCR and 2 kb internal probe P4. (b): Genotype of 12 mice. One band at 0.97 kb was detected in No. 13, 18 and 25; Two bands at 1.2 kb and 0.97 kb were detected in No. 14, 15, 16, 19, 20 and 28; and one band at 1.2 kb was detected in No. 17, 21 and 34. B: Bam HI; S: Sac I; Spe: Spe I; M: Marker; 1833d: MK5-RS-1833d, 5' primer; 2830rc: MK5-RS-2830rc, 3'primer; P4: Spe I-Bam HI fragment, 2 kb internal probe.

(2) Characterisation of the Gene Targeting Mice by Southern Blot Analysis

Mouse-tail genomic DNA were digested by Bam HI and hybridized with probe P4 (an internal probe). The wt signal would be 6.5 kb and the homologous recombination signal would be 2.0 kb (Fig. 3-3). Four mice had been genotyped by SB in Fig. 3-9. No. 270, 251 only had 6.5 kb signal so they were wt (+/+); No. 234 had both 6.5 kb and 2.0 kb signals so it was a MK5 heterozygous (+/-) mouse; No. 232 only had 2.0 kb signal so it was a MK5 ko (-/-) mouse.

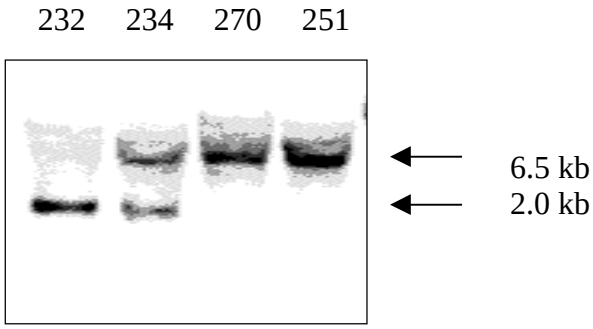


Fig. 3-9: Southern blot analysis of the mice with probe P4.
Mouse-tail genomic DNA was digested by Bam HI then hybridized with 2 kb internal probe P4. One band at 6.5 kb was detected in mouse number 270 and 251; two bands at 6.5 kb and 2 kb were detected in mouse number 234; one band at 2 kb was detected in mouse number 232.

(3) Characterisation of the Gene Targeting Mice by Northern Blot Analysis, RT-PCR and Sequencing

We further characterized the mice on RNA level with Northern blot analysis and sequencing RT-PCR products. Northern blot analysis using MK5 coding domain sequence (MK5 cDNA 707-2128) as a probe (probe P5) detected transcripts both in the total macrophage RNA of wt (+/+) and homozygous ko (-/-) mice (Fig. 3-10). This indicated that the gene targeting does not affect the mutant DNA transcription. To further analyse the total macrophage RNA, we applied RT-PCR with primers MK5-707d and MK5-2128 rc or MK5-988d and MK5-2128 rc. As expected, the PCR products of ko macrophage were about 0.1 kb shorter than the wt products (Fig. 3-11).

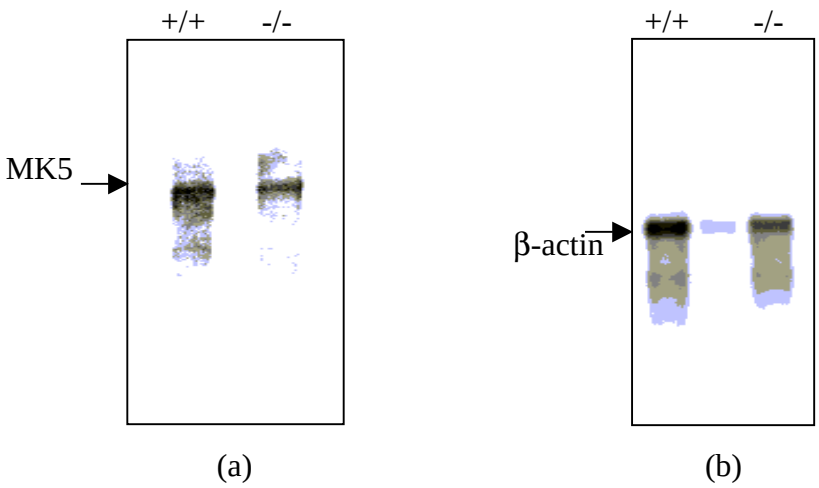


Fig. 3-10: Northern blot analysis of macrophages with probe P5.
Total macrophage RNA of wt (+/+) and MK5 ko (-/-) mice were hybridized with probe P5 (MK5 coding domain sequence, MK5 cDNA 707-2128) (a) or β-actin (b), one band was detected both in wt and MK5 ko mice in (a) and (b).

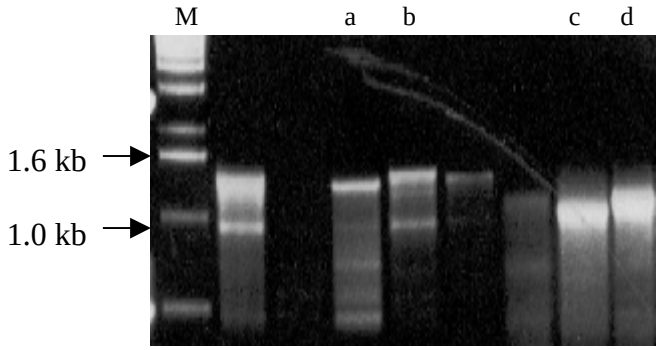


Fig. 3-11: RT-PCR of macrophages
RT-PCR was performed using total macrophage RNA of wt mouse (b, d) or MK5 ko mouse (a, c), with the primer composition of MK5-707d and MK5-2128rc (a, b) or MK5-988d and MK5-2128rc (c, d). One band was detected in lane a, b, c and d. The PCR product in a or c was shorter than that in b or d. M: marker

```
ATGTCGGAGG ACAGCGACAT GGAGAAAGCC ATCAAGGAGA CCTCCATTTT          50
AGAAGAATAT AGTATCAATT GGACTCAGAA ACTGGGAGCC GGAATTAGTG
GTCCAGTTAG AGTCTGTGTG AAGAAATCCA CTCAAGAACG GTTTGCACTG
AAAATTCTTC TTGATCGTCC AAAAGCTAGA AATGAGGAGC GCCTGCACAT         200
GATGTGTGCC ACACACCCCA ACATAGTTCA GATTATTGAA GTGTTTGCTA
ACAGTGTACA GTTCCCTCAT GAGTCCAGCC CCAGGGCTCG ACTTTTAATT
GTTATGGAGA TGATGGAAGG GGGAGAGCTA TTTCACAGAA TCAGCCAGCA
CCGGCACTTT ACAGAGAAGC AAGCCAGCCA AGTAACAAAG CAGGACGCCC         400
CTGTGAAATT ATGTGACTTT GGGTTTGCTA AAGTTGACCA AGGTGATTTG
ATGACACCCC AGTTTACCCC TTACTATGTA GCACCTCAGG TACTGGAAGC
GCAGAGACGG CACCAGAAGG AGAAGTCTGG CATCATACCT ACCTCGCCAA
CACCCTACAC TTACAACAAG AGCTGTGACT TGTGGTCCCT AGGGGTGATA         600
ATTTATGTGA TGCTGTGCGG ATATCCTCCT TTTTACTCCA AACACCATAG
TCGGACTATC CCAAAGGATA TGCGGAAAAA GATCATGACA GGAAGTTTCG
AGTTCCCAGA AGAAGAGTGG AGCCAGATCT CAGAGATGGC TAAAGATGTT
GTGAGGAAGC TTCTGAAGGT CAAACCAGAG GAAAGACTCA CAATCGAGGG         800
AGTGTTGGAC CATCCCTGGC TCAACTCGAC AGAGGCCCTG GATAATGTGC
TACCCTCTGC CCAGCTGATG ATGGATAAGG CGGTGGTTGC GGGGATCCAG
CAGGCGCACG CCGAGCAGCT GGCAAACATG AGGATCCAGG ACCTCAAGGT
CAGCCTCAAA CCCCTGCACT CTGTCAACAA CCCCATTCTC AGGAAGAGGA        1000
AGCTGCTGGG CACCAAGCCA AAGGACGGTA TTTATATACA CGACCATGAG
AATGGAACTG AGGACTCAAA TGTTGCCTTG GAAAAGCTTC GAGATGTCAT
TGCCCAGTGT ATCCTCCCCC AGGCTGGAGA GAATGAAGAT GAGAAGCTGA
ATGAGGTAAT GCAGGAGGCC TGGAAGTACA ACCGCGAATG CAAGCTCCTG        1200
AGGGATGCTC TGCAGAGTTT TAGCTGGAAT GGCCGTGGAT TCACAGATAA
AGTTGACCGA TTGAAGCTGG CAGAGGTGGT AAAGCAGGTG ATCGAAGAGC
AGACCCTTCC CCACGAGCCC CAGTAG                                  1326
```

Fig. 3-12: MK5 ko mice coding domain sequence

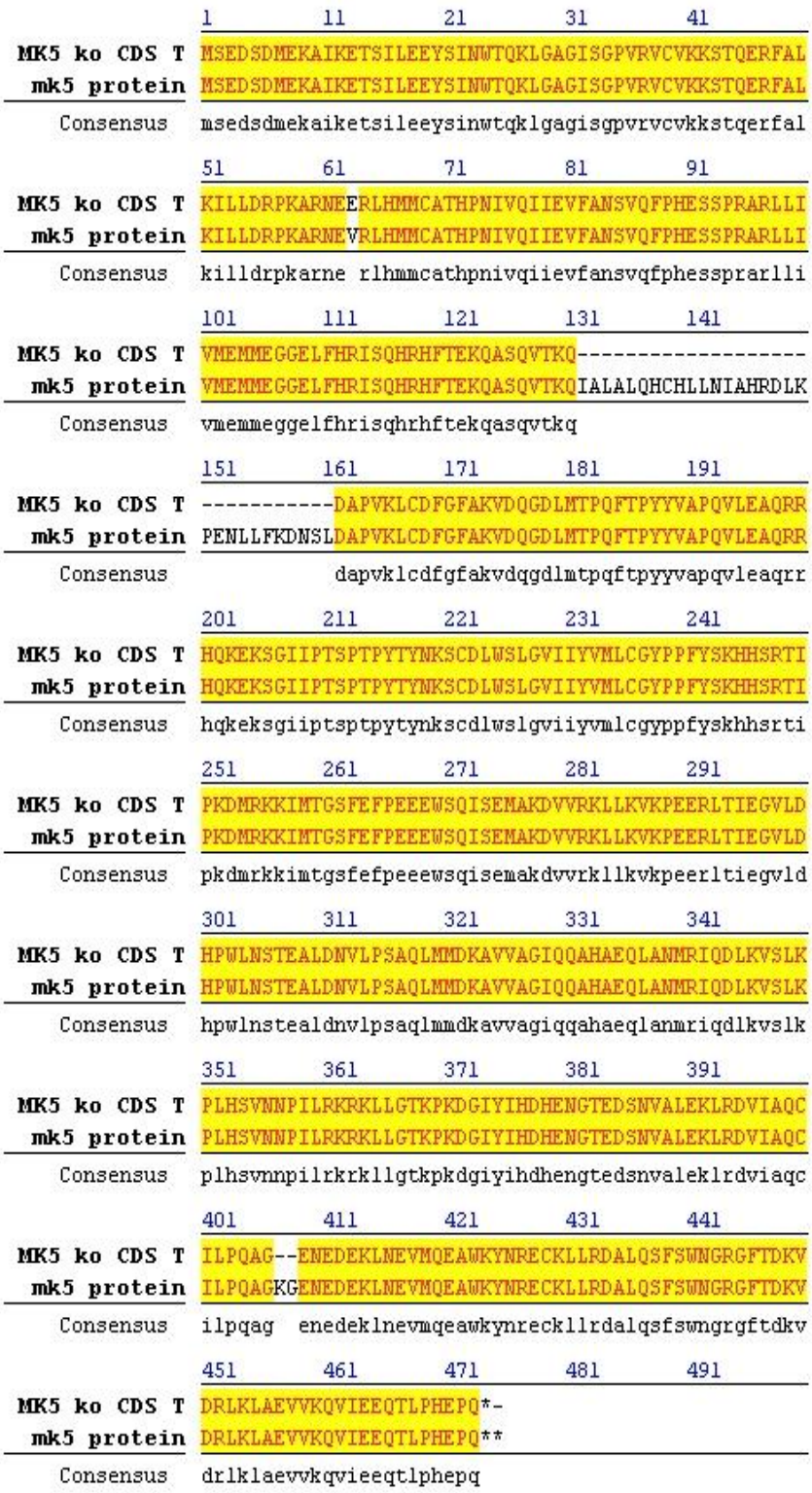


Fig. 3-13: Alignment of MK5 protein sequences of wt and ko
The cDNA of MK5 ko macrophage was mutated by deleting MK5 cDNA of 1100-1189 in the wt (AF039840). This exon deletion removed amino acids 131–161 of MK5. Amino acid number 63 in wt was valine (V) while in ko was glutamic acid (E); and another two more amino acids, lysine and glycine (K and G), number 407 and 408, were also missing in ko protein sequence.

Cloning and sequencing of the PCR product of ko macrophage cDNA with primers MK5-707d and MK5-2128 rc, we found that the cDNA of ko macrophage was mutated by deleting MK5 cDNA of 1100-1189 in the wt which stands for exon 6. This exon deletion removed amino acids 131–161, "**IALALQHCHLLNIAHRDLKPENLLFK DNSL**", which consist of the conserved kinase subdomain VI a and VI b. Since subdomain VI b contains the catalytic region of MK5 required for protein kinase activity, the ko mice should not have MK5 kinase activity. This was confirmed by the kinase assays of MK5 later. After the deletion, MK5 ko coding domain sequence was still in the reading frame (Fig. 3-13). This could explain why we still detected the MK5 signal in ko mouse in Northern blot analysis using MK5 coding domain sequence as a probe. There were another two more differences between the wt and ko MK5 protein sequences: (1) Amino acid number 63 in wt was valine (V) while in ko was glutamic acid (E). This maybe because of the mismatch of PCR, G**T**G (wt) to G**A**G (ko), which stands for V or E. (2) another two more amino acids, lysine and glycine (K and G), number 407 and 408 were also missing in ko protein sequence. This may result from the individual difference between the two mice line.

(4) Characterisation of the Gene Targeting Mice by Western Blot Analysis, Protein Kinase Assay and Subcellular Localization

To determine whether the mutant mice still have MK5 kinase activity, we further characterized the mice by Western blot analysis and kinase assay. MK5 ko MEFs were prepared from five E13.5 day MK5 ko embryos. Proteins were extracted from the same amount of (0.2×10^5) cells. Extracts of MEF and macrophage from wt (+/+) or MK5 ko (-/-) animals were examined by Western blot analysis with a polyclonal antibody directed against MK5 (anti-PRAK, Upstate). Anti-PRAK is a sheep immunoaffinity purfied IgG which used a full length human PRAK as antigen, it also contains a N-terminal hexa-histidine tag and was expressed in Sf 9 cells. The Western blot analysis confirmed the absence of 54 KDa MK5 protein both in MEF and in macrophage of MK5 ko animals (Fig. 3-14). From the sequence of RT-PCR product, we know that the mutant MK5 protein was 30 amino acids shorter than the wt MK5 protein. The weak lower molecular weight band could be detected in Western blot, which corresponds probably the truncated kinase.

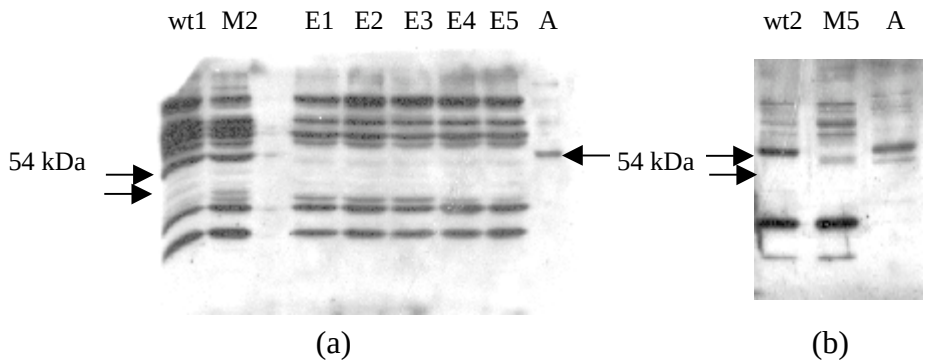


Fig. 3-14: Western blot analysis
Extracts of MEF and macrophage from wt (+/+) or MK5 ko (-/-) mice were examined with anti-PRAK antibodies (Upstate). 54 KDa signal was absent in E1 to E5 (a) and M5 (b), but a weak lower molecular weight band could be detected in these lanes. wt1: wild type MEF; M2: MK2 ko MEF; E1 to E5: MK5 ko MEF from 13.5 day embryo 1 to 5; A: A 431 cell lysate, positive control of MK5, 54 KDa; wt2: wild type macrophages

In the next step, we analysed MK5 kinase activity. MEFs were stimulated by treatment with 250 µM arsenite or 300 mM sorbitol for 60 min. Proteins were extracted from the cells and kinase was immunoprecipitated from the cell lysates with anti-PRAK antibody (Upstate). Kinase assays were performed using PRAK-substrate peptide (Upstate). Both wild type and MK2 ko MEFs showed basal kinase activities, more than 1250 cpm, while the kinase activity was hardly detected in MK5 ko MEF (protein kinase assays demonstrated that the MK5 ko (-/-) MEF was deficient in MK5 activity (Fig. 3-15).) The differences of kinase activity between wt MEF and MK5 ko MEF were significant (P<0,01), but there were no differences of the kinase activity between all the stimulated and non-stimulated MEFs (P>0,05) (Fig. 3-15).

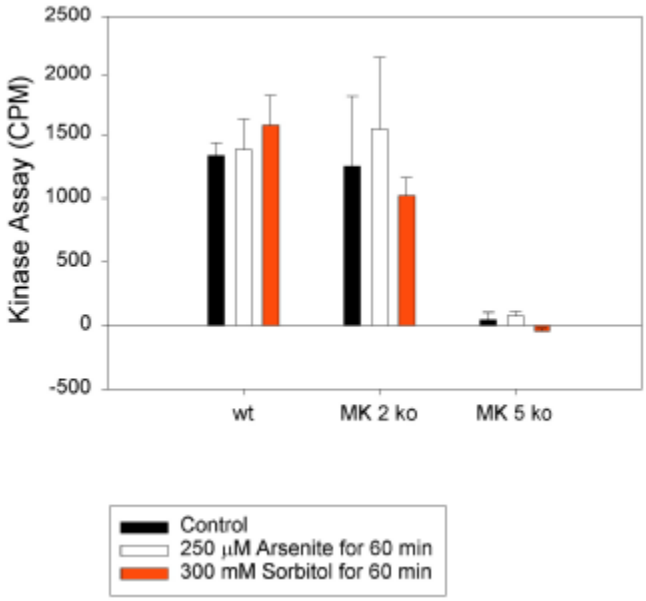


Fig. 3-15: MK5 kinase assay in MEFs
MEFs were stimulated by treatment with 250 µM arsenite or 300 mM sorbitol for 60 min. Proteins were extracted from the cells and kinase was immunoprecipitated from the cell lysates with anti-PRAK antibody (Upstate). Kinase assays were performed using PRAK-substrate peptide (Upstate). The kinase activity was measured by scintaillation. Wt: wild type MEF; MK2 ko: MK2 ko MEF; MK5 ko: MK5 ko MEF.

To confirm the experimental system for kinase assay works well, we performed MK2 kinase assays with the same cell lysates above and detected a very strong activation in MK2 kinase assays (Fig. 3-16). The kinase assays were performed similar to the MK5 kinase assay. In this case we used 5µl anti-MK2 antibody (prepared by our group) to immunoprecipitate MK2 from the same cell lysates (for MK5 kinase assay above). In all non-stimulated MEFs, the kinase activity was very low, the signal was in range between 360 to 630 cpm. MK2 activity could be highly activated by arsenite or sorbitol both in wt and MK5 ko MEFs. Stimulated by arsenite treatment for 60 min, MK2 activity reached relative level of 8000 cpm in wt MEF and 15200 cpm in MK5 ko MEF. The differences of the kinase activity between non-stimulated cells and cells stimulated by arsenite were significant both in wt MEF (P<0,001) and in MK5 ko MEF (P<0,05). After hyperosmotic treatment with sorbitol for 60 min, the relative kinase activity reached 1190 cpm in wt MEF and 3600 cpm in MK5 ko MEF. In contrast, in MK2 ko MEFs, neither arsenite nor sorbitol could activate the kinase and there were no difference of the kinase activity between the stimulated and non-stimulated MK2 ko MEFs (P>0,05) (Fig. 3-16).

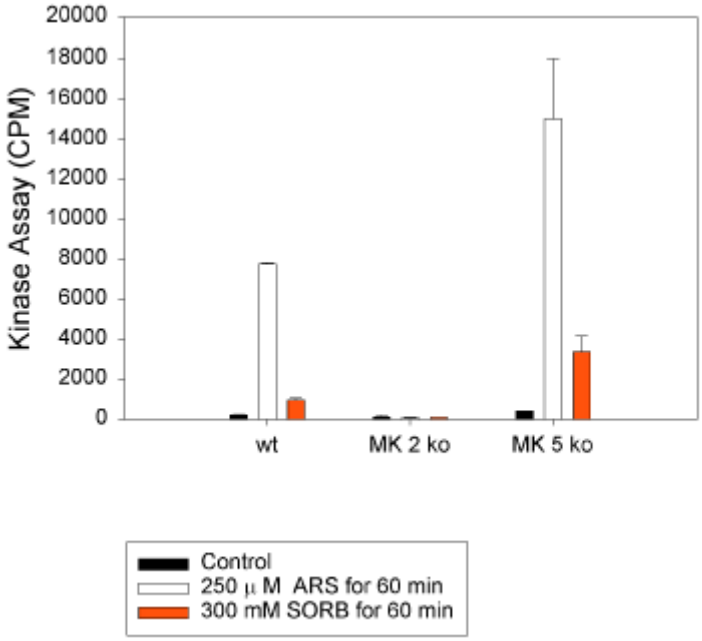


Fig. 3-16: MK2 kinase assay in MEFs
MEFs were stimulated by treatment with 250 μM arsenite or 300 mM sorbitol for 60 min. Proteins were extracted from the cells and kinase was immunoprecipitated from the cell lysates with anti-MK2 antibody (prepared by our group). Kinase assays were performed using PRAK-substrate peptide (Upstate). The kinase activity was measured by scintaillation. Wt: wild type MEF; MK2 ko: MK2 ko MEF; MK5 ko: MK5 ko MEF.

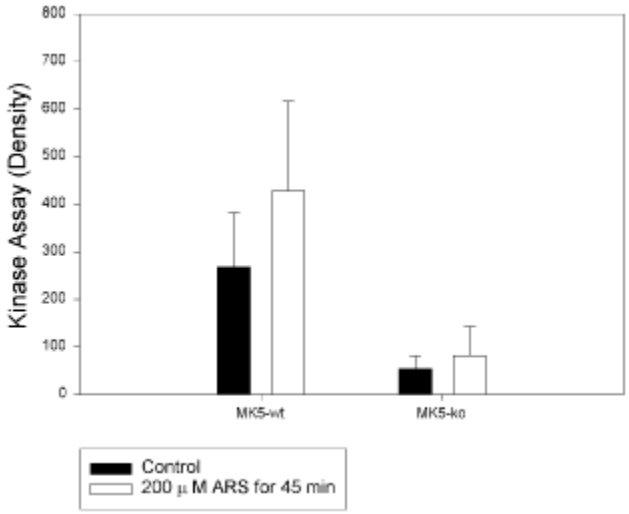


Fig. 3-17: MK5 kinase assay in transfected MEFs
Transfected MEFs were stimulated by treatment with 200 μM arsenite (ARS) for 45 min, Proteins were extracted from the cells and kinase was immunoprecipitated from the cell lysates with anti-PRAK antibody (Upstate). Kinase assays were performed using PRAK-substrate peptide (Upstate). The kinase activity was measured by the spot density using the phosphoimaging. MK5-wt: MK5 ko MEF transfected with pEGFP and pSG5-myc-MK5-wt expression vectors; MK5-ko: MK5 ko MEF transfected with pEGFP and pcDNA4-His-Max-MK5-ko expression vectors.

In order to determine the activator of MK5 and the function of mutant MK5 protein, we co-transfected pEGFP and the pSG5-myc-MK5-wt expression vectors or pEGFP and pcDNA4-His-Max-MK5-ko expression vectors to MK5 ko MEF and then measured the MK5 kinase activities of the transfected cells. We obtained similar results in MK5 kinase assays. The transfection efficiency was reached to 60% in all experiments, checked by fluorescence microscopy. Cells were stimulated by 200 μM arsenite for 45 min. MK5 was immunoprecipitated from the same amount of proteins as above with anti-PRAK antibody (Upstate), then the kinase assays were carried out under the same conditions as MK5 kinase assay described above. The kinase activity was measured by the spot density using the phosphoimaging. The kinase activity of MEF transfected with MK5-wt expression vector was about 370 and after stimulation, no significant difference increase was detected (P>0,05). The kinase activity of MEF transfected with MK5-ko expression construct was about 150 and after stimulation, no significant difference was detected neither (P>0,05). The comparison of activities between the MEFs transfected with MK5-wt and MK5-ko vectors, both in non-stimulated and stimulated by arsenite cells, exhibits no significant difference (P>0.05) (Fig. 3-17). These results showed that there were no activation of MK5 even in the transfected cells and the mutant MK5 had no kinase activity.

Taken together, the results demonstrate that the targeted disruption of the MK5 gene resulted in a null allele.

Subcellular localization of p38 MAPK and their downstream targets of p38 MAPKs may show their different physiological functions in certain conditions. For mouse MK2, two regulatory phosphorylation sites, T205 and T317, have been identified (Engel et al., 1995). T205 is located in the kinase activation loop between subdomains VII and VIII whereas T317 is outside the catalytic domain in a hinge region between the catalytic domain and the C-terminal extension, which carries an autoinhibitory A-helix motif, the nuclear export signal (NES) and the nuclear localization signal (NLS) (Engel et al., 1998). Under control conditions, MK2 is present in the nucleus, and upon stimulation it becomes phosphorylated by p38 and is consequently exported from the nucleus probably due to unmasking of its NES. This translocation is blocked by SB203580 and leptomycin B (LMB) (Ben-Levy et al, 1998; Engel et al, 1998). Interestingly, the mutation T317E, but not T205E, resulted in a protein constitutively exported from the nucleus and accumulated in the cytoplasm of the cell (Ben-Levy et al., 1998; Engel et al., 1998). Hence, it is supposed that phosphorylation of T317 is the critical event for parallel regulation of MK2 activity and localization.

The export and conformation of MK2 were also studied by fusion of green fluorescent protein (GFP) variants to the N- and C-terminus. An expression plasmid EGFP-MK2-EBFP (GMB) was constructed. It has been found that GMB is present in the nucleus of non-stimulated Swiss 3T3 cells and translocates to the cytoplasm after stress stimulation which reflects the major properties of wt MK2. When Crm1-dependent nuclear export is blocked by LMB, GMB accumulates in the nucleus. Expressed GMB or the mutant GMB-T317E in HEK293 cells and analysed by fluorescence resonance energy transfer (FRET), it has been found that GMB exists in the closed (an inactive) conformation in the nucleus and GMB-T317E has an open (an active) conformation in the cytoplasm. This indicated that in living cells activation of MK2 and its nuclear export are coupled by a phosphorylation-dependent conformational switch. (Neininger et al., 2001).

Following phosphorylation of MK2, nuclear p38 is exported to the cytoplasm in a complex with MK2. The cytoplasm translocation of MK2 requires phosphorylation by p38 without a requirement for MK2 activity (Ben-Levy et al., 1998). MK2 serves both as an effector substrate and as a determinant of cellular localization of p38. Nuclear export of p38 and MK2 may permit them to phosphorylate cytoplasmic substrates, such as eukaryotic initiation factor (eIE)-4E, PHAS-1 and Hsp25/27.

Since we could not measure the activation of MK5 in kinase assays even in the transfected cells (Fig. 3-17), we further studied the subcellular localization of MK5 in response to the stimulation of arsenite in transfected Hela cells try to find some cell biological changes after stimulation. Hela cells were transfected with pEGFP-MK5 or co-transfected with pEGFP-MK5 and pcDNA3-HA-p38 vectors. Transfected cells were treated with or without SB203580 for 30 min, stimulated by 200 M arsenite. Export of MK5 was analysed by fluorescence microscopy. It has been found that in the case of p38 co-transfected Hela cells, after stimulation by arsenite for 90 min, some MK5 export can be found (Fig. 3-18). The export result corresponds with the kinase assay result, no export (no activation) could be found in Hela cells only transfected with MK5-wt expression vector even after 90 min. This further indicated that arsenite is not the suitable activator of MK5.

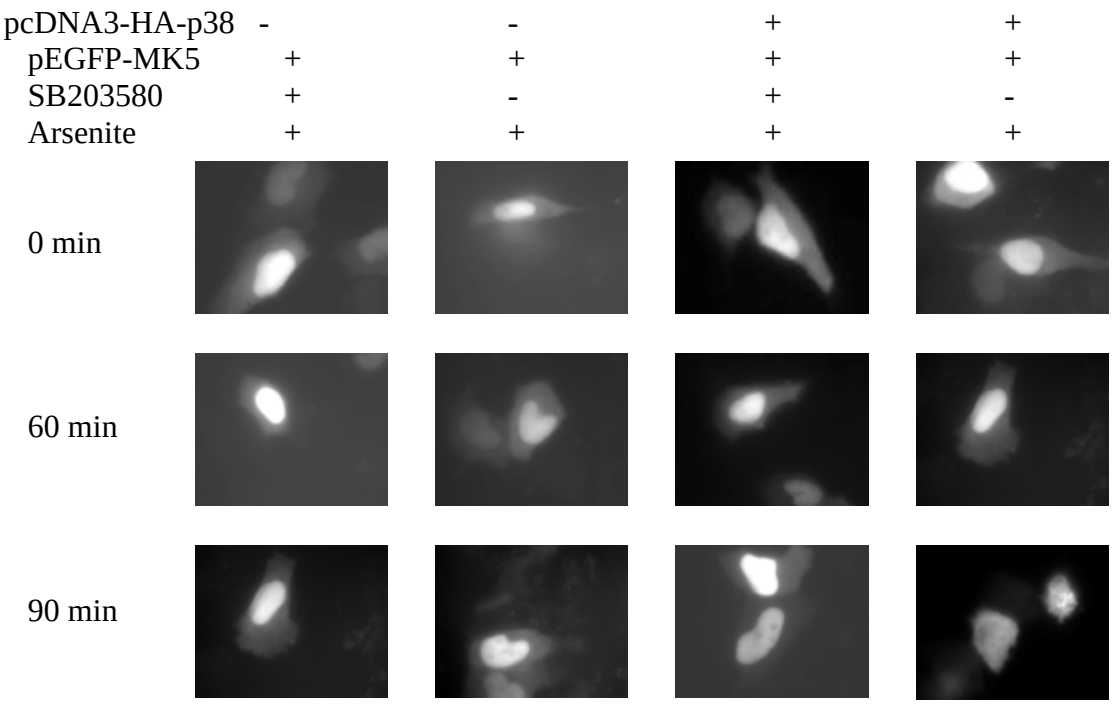


Fig. 3-18: Export of MK5
Hela cells were transfected with pEGFP-MK5 or co-transfected with pEGFP-MK5 and pcDNA3-HA-p38 vectors. The transfected cells were treated with or without SB203580 for 30 min. The export of MK5 was analysed by fluorescence microscopy at 0, 60 and 90 min after stimulation by 200 M arsenite. Only in the case of p38 co-transfected Hela cells, after stimulation by arsenite for 90 min, some MK5 export could be found. No export of MK5 was found in other transfected cells.

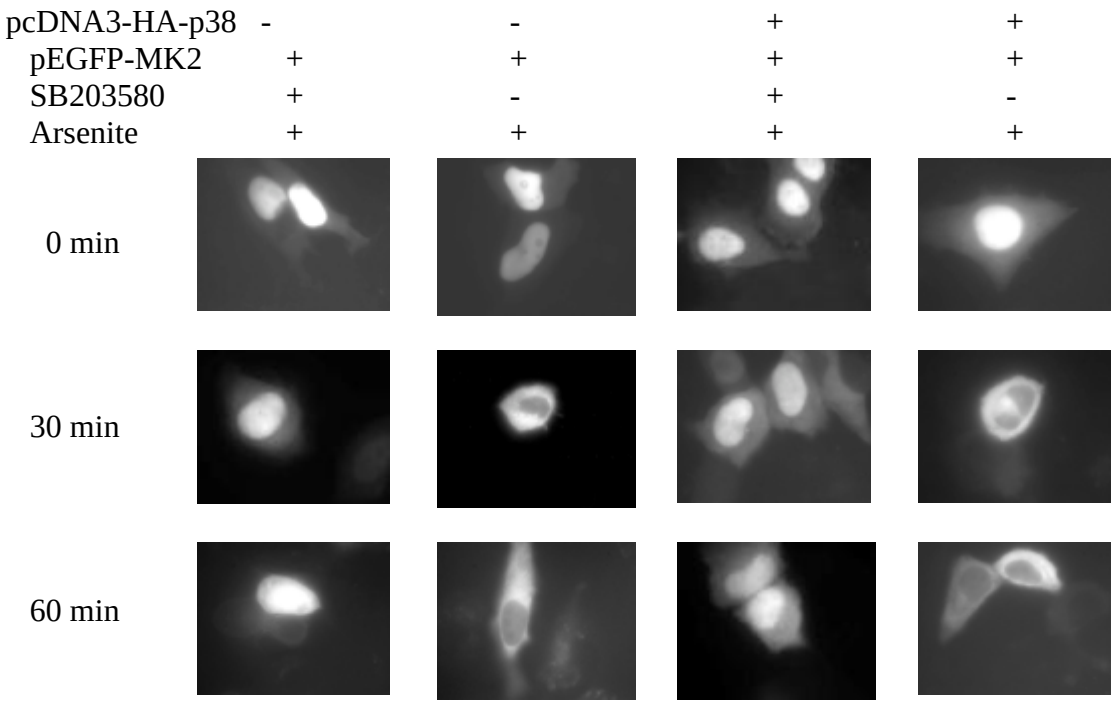


Fig. 3-19: Export of MK2
Hela cells were transfected with pEGFP-MK2 or co-transfected with pEGFP-MK2 and pcDNA3-HA-p38 vectors. The transfected cells were treated with or without SB203580 for 30 min. Export of MK2 was analysed by fluorescence microscopy at 0, 30 and 60 min after stimulation by 200 M arsenite. The export of MK2 could be found after stimulation by arsenite for 30 min and there were no difference on exporting between pEGFP-MK2 transfected cell and the p38 co-transfected cells.

In order to compare MK5 with MK2, we also studied the subcellular localization of MK2 in the transfected Hela cells. Hela cells were also transfected with pEGFP-MK2 or co-transfected with pEGFP-MK2 and pcDNA3-HA-p38 vectors. The export of MK2 could be found after stimulation by arsenite for 30 min and there were no difference on exporting between pEGFP-MK2 transfected cell and the p38 co-transfected cells (Fig. 3-19). Compared with MK2, the export of MK5 was far from being complete and slower.

MK5 differs from MK2, with one regulatory phosphorylation site, T182. Like MK2, MK5 also has a functional NLS and a NES. Prof. Ugo Moens and Dr. Ole Morten Seternes analysed MK5 export. They found that “361RKRK364” of MK5 are involved in the functional NLS and three “L” in “345LJVSLKPLHS354” motif are related to the functional NES. Treatment of cells with arsenite results in nuclear export of MK5. Time course studies showed that this transport was much slower then MK2. Sorbitol induced nuclear export of MK2, but not of MK5. Arsenite-induced export of MK5 is inhibited by leptomycin B (Prof. Ugo Moens and Dr. Ole Morten Seternes’ observation, submitted). Our export results are comparable with theirs.

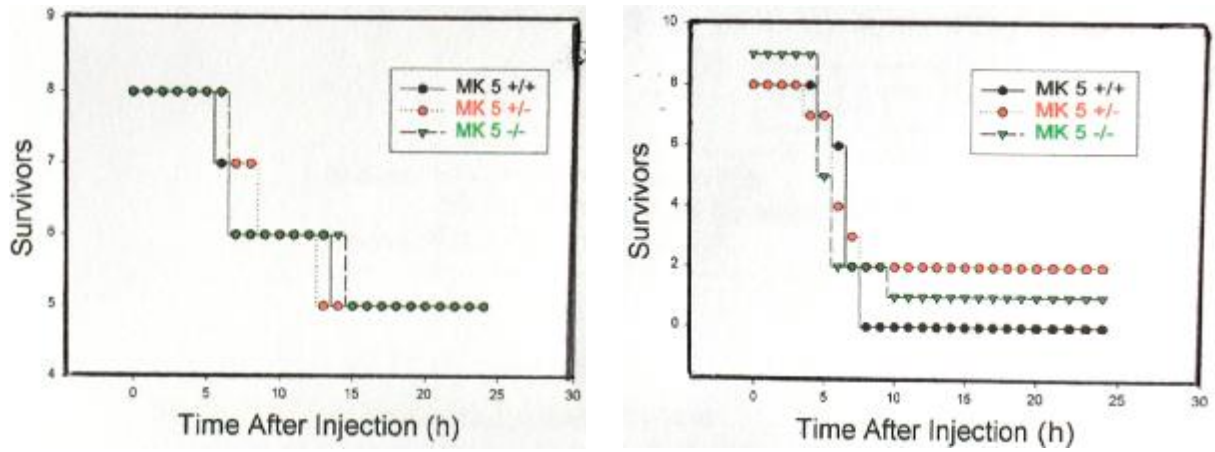
Both kinase assay and export results suggest that though there are some phylogentic conservation between MK2 and MK5 (Fig. 3-1), MK5 is quite different from MK2 which is considered as the main direct downstream substrate of p38. Arsenite, sorbitol which known as the activators of p38 MAPKs could not activate MK5 in our experiments. It has been reported that PRAK, the human homologues of murine MK5, could be activated by arsenite and PMA. (New et al, 1998). When we used the same antibody

(this anti-PRAK provided by professor Jiahuai Han) to do the MK5 kinase assay, we could detect the activation in wt MEF, but not in MK2 ko MEF (data not shown). This indicated that this antibody was not specific enough and immunoprecipitated MK5 and MK2 at the same time. Hence, the kinase activity measured in citated experiments was probably due to a contamination of MK2 kinase activity. Since MK2 is much more active toward this substrate than MK5, MK2 kinase activity could superimpose MK5 kinase activity.

Very recently, a new autoactivation pathway of p38α has been reported. It has been found that p38α can be activated by autophosphorylation (Ge et al, 2002). This indicates that there are some pathways of p38 MAPKs which are still unknown. In our MK5 kinase assay and export experiments, we could not activate MK5 kinase with some activators already known for p38 MAPKs, there are two possibilities to explain this: (1) p38-cascade had more alternative pathways and the activation conditions for p38 MAPKs to redirect the signal towards MK5 have not been found yet. (2) An alternative possibility would be that MK5 is not or mainly not activated by p38 MAPK cascade *in vivo*. Future work needs to be done to elucidate the mechanism of the activation of MK5 *in vivo.*

3. Analysis of MK5-Deficient Mice

MK2 ko mice show increased stress resistance and survive in LPS-induced endotoxic shock which is due to a reduction of about 90% of TNF-α (Kotlyarov et al., 1999). To analyse the MK5 deficient mice, therefore we firstly examined whether MK5 ko mice response to LPS-induced endotoxic shock in the same way. Endotoxic shock was induced by LPS and D-galactosamine. The mice were challenged with intraperitoneal administration of 1 µg of LPS and 20 mg of D-galactosamine or 10 µg of LPS and 20 mg of D-galactosamine. In the lower dosage group, 8 of each genotype mice were subjected to endotoxic shock. The first mouse died at 6 h after administration and each genotype still had 5 survivors after 24 h (Fig. 3-20, a).



(a) (b)

Fig. 3-20: Effect of LPS/D-galatosamine on the survival in MK5 mutant mice
(a): Survival of wild type (+/+), MK5 (+/-) and MK5 (-/-) mice after treatment with 1 µg of LPS and 20 mg of D-galactosamine. (b): Survival of wild type (+/+), MK5 (+/-) and MK5 (-/-) mice after treatment with 10 µg of LPS and 20 mg of D-galactosamine.

In the higher dosage group, 8 MK5 +/+ and +/-, 9 MK5 -/- mice were subjected to the endotoxic shock. The mice started to die after 4 h, two MK5 +/- and one MK5 -/- mice still survived after 24 h (Fig. 3-20, b). There were no differences in surviving among the three different MK5 genotype (+/+, +/- and -/-) mice respond to LPS-induced endotoxic shock.

We further measured the production of the cytokines in spleen cells induced by LPS, staphylococcal enterotoxin (SEB) or concanavalin A (Con A). The result is shown in Fig. 3-21. We found that TNF-α production was not defective in MK5 ko mice, this could explain why there were no differences in surviving among the three different MK5 genotype mice in the LPS-induced endotoxic shock model. Production of IL-4 in spleen cells stimulated by Con A seems to be defective in MK5 mutant mice in this experiment, about 75% has been inhibited (P<0,05). The production of IL-12, both p40 and p70, shows a tendency to increased levels. Details are shown in Table 3-5.

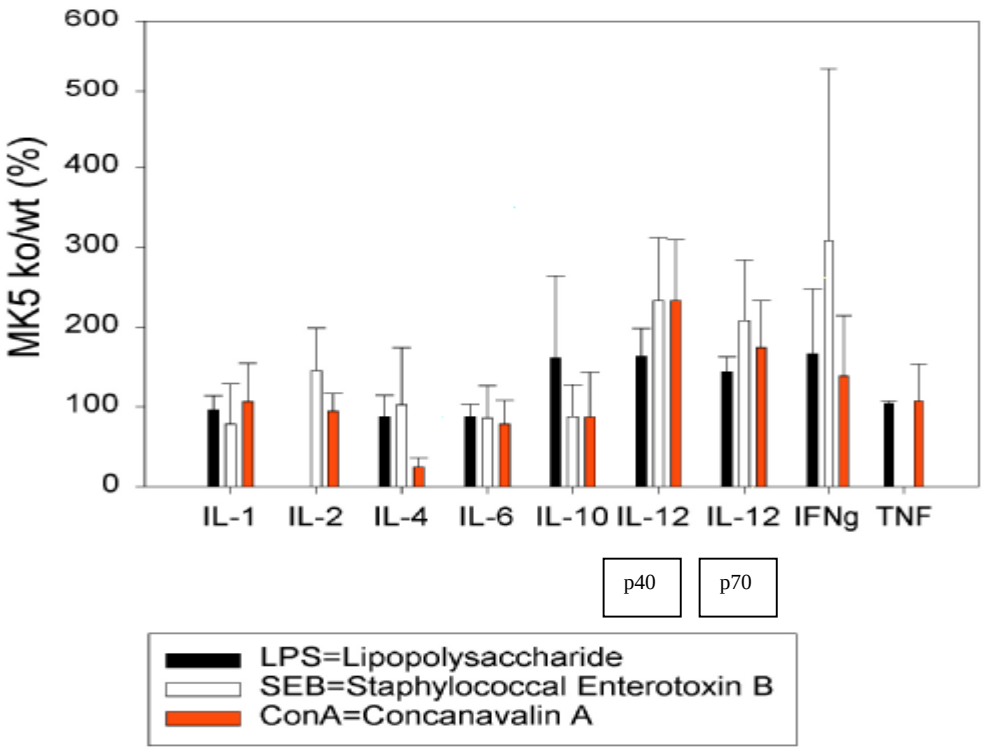
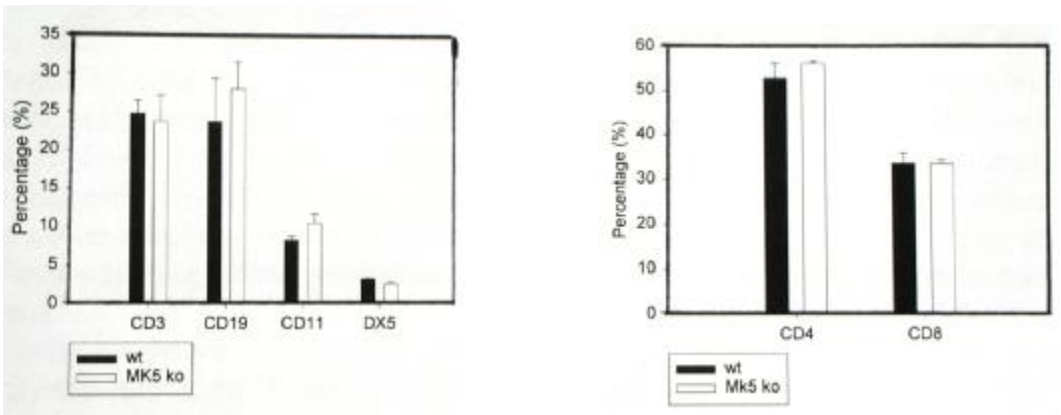


Fig. 3-21: Cytokines production by spleen cells
Spleen cells from wild type and MK5 ko mice were stimulated by LPS, staphylococcal enterotoxin (SEB) or concanavalin A (Con A). The cytokines were determined with an ELISA kit (Quantikine™, R&D Systems).

Table 3-4: Production of IL-4 and IL-12 of spleen cells derived from wt (+/+) and MK5 ko (-/-) mice

Cytokines	Mice and Re	Sti.	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Average
IL-4	wt	LPS	32±4	12±11	30±17	0	0	
		SEB	780±244	57±21	139±50	43±4	7±422	
		ConA	1000	307±55	423±101	418±70	42±124	
	Mk5 ko	LPS	24	15+-13	19+-11	0	0	
		SEB	32±7	39±14	99±23	84±9	129±34	
		ConA	101±6	78±19	101±16	121±39	185±24	
	Re: ko/wt (%)	LPS	75	125	63			87.7±26.8
		SEB	4	68	71	195	174	102.4±71.5
		ConA	10	25	24	21	44	24.8±10.0
IL-12 (p40)	wt	LPS	22±027	416±12	18±134	877±375		
		SEB	170±21	289±11	301±51	816±290		
		ConA	172±15	126±13	146±29	71±4315		
	Mk5 ko	LPS	420±15	837+-82	262±44	1005±270		
		SEB	495±41	956±74	468±129	1270±133		
		ConA	433±60	441±16	224±52	1275±494		
	Rel: ko/wt (%)	LPS	191	201	145	115		163±34.8
		SEB	290	330	155	156		232.8±78.5
		ConA	250	350	153	179		233±76.3
IL-12 (p70)	wt	LPS	36	63±13	42±16			
		SEB	29	32±25	54±16			
		ConA	21	69±16	56±22			
	Mk5 ko	LPS	55±38	103±48	50±12			
		SEB	49±19	10±054	75±40			
		ConA	54±21	94±38	72±15			
	Rel: ko/wt (%)	LPS	150	163	118			143.7±18.9
		SEB	170	313	140			207.7±75
		ConA	257	136	128			173.7±59



(a) (b)

Fig. 3-22: Leukocyte antigens
(a) Spleen cells from wild type and MK5 ko mice were examined with CD3, CD11, CD19 and DX5 antigens. (b) T cells from wild type and MK5 ko mice were examined with CD4 and CD8 antigens. wt: wild type mice; MK5 ko: MK5 ko mice.

Cytokine production also depends on the cell type. Therefore we checked the leukocyte antigens and found that in MK5 ko mice, no significant change in the numbers of monocytes/ marcrophages (CD11b), Pan T cells ($CD3^+$), Pan B cells ($CD19^+$), NK cells (DX5), helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) was observed on analysis of spleen cells and T cells (Fig. 3-22). This indicated that the differences among cytokine production were not because of the differences of cell types and normal differentiation and maturation of blood cells.

In human peripheral leukocytes and lymphoid organs, p38α mRNA expression is relatively high, p38δ expression is intermediate, and expression levels of p38ß and p38γ are low (Wang et al., 1997). p38 MAPKs may be involved in the production of various cytokines at multiple levels of regulation. $CD4^+$ Th cell responses can be divided into distinct effector classes, Th1 or Th2, defined by the selective production of either IL-2 and IFN-γ, which primarily promote cell-mediated immunity (Th1), or IL-4 and IL-5, which promote IgE production and eosinophilia (Th2) (Constant & Bottomly, 1997). Of these two classes, the development of the Th2 response is especially dependent on CD28 costimulation. In several studies, human $CD4^+$ T cells stimulated *in vitro* without CD28 costimulation developed a Th1 phenotype, producing only IL-2 and IFN-γ, whereas the addition of an anti-CD28 Ab to the culture converted the population to a Th2 phenotype, inducing production of IL-4 and IL-5. (King et al., 1995; Webb and Feldmann, 1995; Kalinski et al., 1995). It has been shown that CD28 ko mice exhibited greatly reduced IL-4, IgE, and IgG1 production in response to anti-IgD Abs (Gause et al., 1997), this indicated that particular Th2 responses may vary in their dependence on CD28. For MK5 ko mice, further work with CD28 costimulation needs to be done and in this way, maybe we can find some activators of MK5.

It has been also reported that the production of IL-5, IL-6, IL-9, IL-10 and IL-13 are impaired in IL-4 ko mice because IL-4 is required for the generation of Th2-derived cytokines, and therefore, immune responses dependent on these cytokines become impaired (Kopf et al., 1993; Kopf et al., 1995). In MK5 ko mice, the release of IL-10 was not affected (Fig. 3-21; Table 3-5). Future work might identify whether MK5 ko mice are defective on production of some other Th2-derived cytokines. It has been demonstrated that the p38 MAPK pathway is necessary for IFN-γ production by Th1 effector T cells without affecting IL-4 production by Th2 cells (Rincon et al., 1998). In both Th1 and Th2 cells, CD28 signalling activated p38 and was required for cytokine production. In polarized Th1 and Th2 cell lines, SB 203580 strongly inhibited IL-4 production by Th2 cells, but only partially inhibited IFN-γ and IL-2 production by Th1 cells (Schafer et al., 1999). MK2 ko mice show markedly defective on production of TNF-α and IFN-γ (Kotlyarov, 1999) while MK5 ko mice are defective on production of IL-4. This indicated that MK2 ko mice are defective on Th1 effector T cells whereas MK5 ko mice are maybe defective on Th2 effector T cells. MK2 and MK5 may stand different functions on cytokines production in p38 MAPK pathway. Future studies using the MK2 and MK5 double ko mice, we can make further demonstration of the Th effector T cells. Therefore, the functions of MK2 and MK5 on cytokine production are different. By this means, MK5 maybe also serve as a promising target for specific anti-inflammatory therapy.

Germinal centers (GCs) are known as compartments for B cell differentiation and proliferation. After stimulation of antigen (Ag), GC B cells differentiate into memory cells or plasma cell precursors (Liu et al., 1996). Production of plasma cell precursors and maintenance of serum Ab levels are closely related to GC function (Wu et al.,

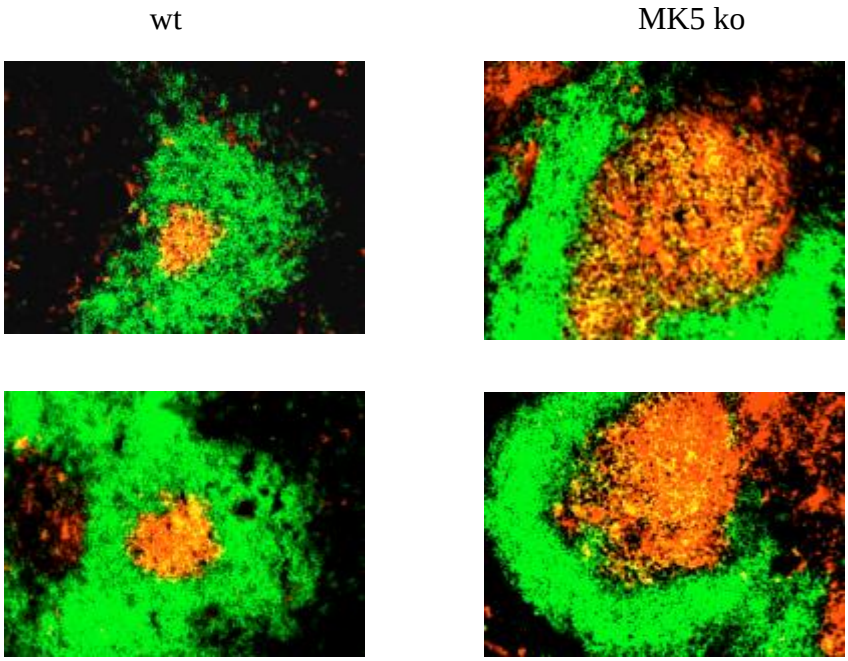
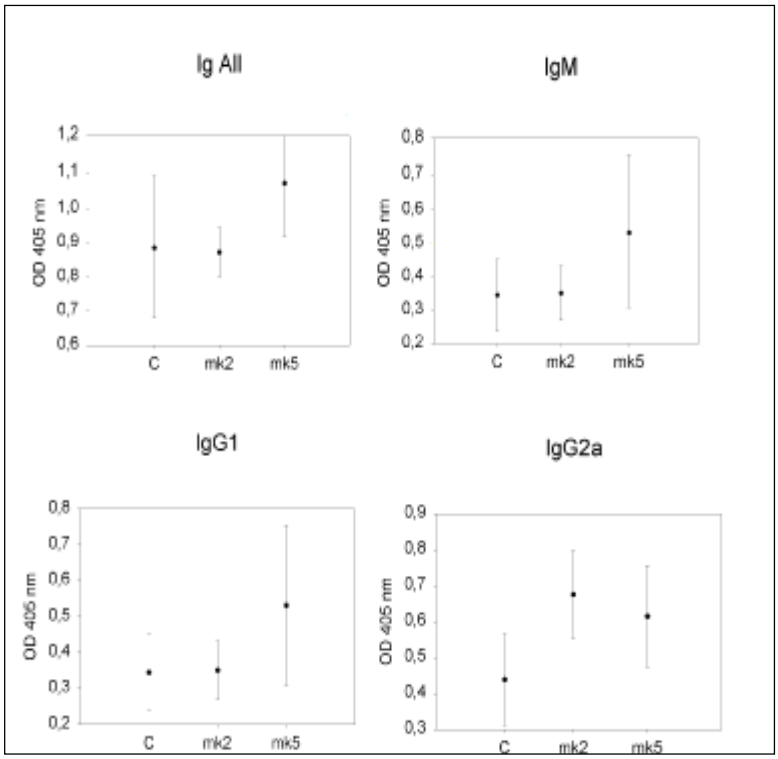


Fig. 3-23: Germinal centre formation in spleens of wt and MK5 ko mice after immunization for 12 days.
After immunization with human serum albumin coupled to DNP in incomplete Freunds adjuvant (IFA) for 12 days, the mice were killed and the germinal centres in the spleen were examined. The centrocytes (red) were stained by rhodamine-labelled peanut agglutinin (PNA) and the B-cells (green) were co-stained by FITC-B220-pan-B. wt: wild type mice; MK5 ko; MK5 ko mice.

1996). Therefore, the kinetics of GC size and number should be closely related to the magnitude and characteristics of humoral immune response. Our observation revealed that after immunization, the mice were able to form germinal centres, but the germinal centres in MK5 ko mice were significantly enlarged (Fig. 3-23). Furthermore, the Ab production in wt and MK2 ko mice was increased after secondary Ag challenge, but in MK5 ko mice this production was unchanged (Fig. 3-24). Similar result of the abnormal germinal center formation was reported to be found also in IL-4 ko mice. In comparison with wt mice, lymph nodes (LNs) of IL-4 ko mice on days 4 and 7 after final Ag challenge were larger and contained a markedly greater number of GCs, which showed marked size variations with a large number of small GCs and a small number of markedly large GCs. By day 14, the number of GCs decreased to the same level as that in wt mice. However, the LN size in IL-4 ko mice was still larger than that in wt mice due to the presence of markedly large GCs (Andoh et al., 2000). It has been also reported that the complete absence of GC formation in Peyer's patches of IL-4 ko mice after oral immunization (Vajdy et al., 1995).

IL-4 is very important in GC B cell differentiation (Cerutti et al., 1998) and in B cell-FDC (Follicular dendritic cell) interaction (Tew et al., 1997). IL-4 production is defective in MK5 ko mice which may result in, at least partly, the abnormal germinal center formation. The same abnormal germinal center formation was also found in MK2 ko mice and which is probably due to the decreased apoptosis of cells in germinal center (Kotlyarov, 1999). This indicates that MK2 and MK5 may share an overlapping function or have a common downstream substrate on transducing apoptosis signal in p38 MAPK cascades.

(a)



(b)

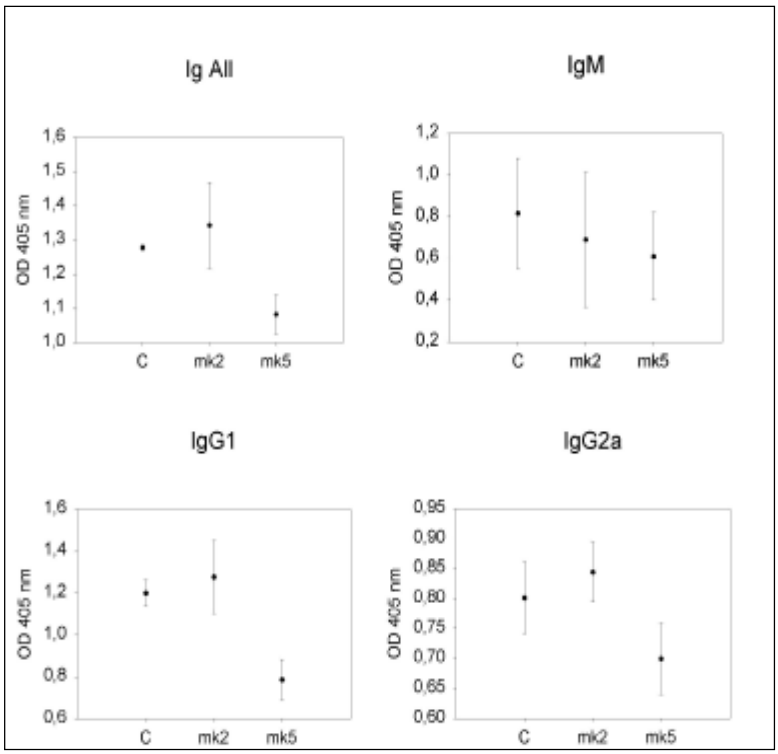


Fig. 3-24: Production of immunoglobulins after immunization
Sex-matched animals, 6-12 weeks old, were immunized by intraperitoneal administration of 100 µg of human serum albumin coupled to DNP in incomplete Freunds adjuvant (IFA). Serum samples were collected from the mice10 days after primary immunization (a) or secondary immunization (b). Levels of DNP-specific immunoglobulin (Ig) isotype were determined by ELISA (Southern Biotechnology Associates). Anti-mouse Ig capture antibodies were used for the measurement of non-specific antibodies level. c: control; mk2: MK2 ko mice; mk5: MK5 ko mice.

4. Further Analysis of MK5-Deficient Mice

Although MK5/PRAK was found three years ago, we still know very less about this kinase. To study the role of the p38 MAPK pathway, and the function of MK5 *in vivo*, we have generated mice with a germline mutation of the MK5 gene. The mice have been characterised by Southern blot analysis, RT-PCR, sequencing, Western blot analysis and protein kinase assays which all demonstrated that the targeted disruption of the MK5 gene resulted in a null allele.

MK5-deficient mice were viable and fertile. The intrinsic MK5 kinase activity is absent in MK5 deficient mice and could be detected in wt mice, but we were not able to activate this kinase with typical p38 pathway stimuli in our experiments. The further analysis of MK5 deficient mice would be firstly to find the proper activator of this kinase and to elucidate the signal transducing pathway of MK5. It is also very important to find the biological relevant substrates of MK5 and to demonstrate if Hsp25/27 is a substrate of MK5 *in vivo*.

Analysis of cytokine production revealed a defect in the production of IL-4 in spleen cells stimulated by Con A in MK5 mutant mice in our experiment. The production of IL-12, both p40 and p70, shows a tendency to increased levels. The defect of MK5 deficient mice in the production of IL-4, could result in an impaired Th2 $CD4^+$ immune response. Future work might identify that if MK5 ko mice are also defective on production of some other Th2-derived cytokines. It would be also interesting to know whether the regulatory mechanism involves the transcriptional or post-transcriptional processes.

p38 MAPK cascade regulates gene expression, correlates with apoptosis and controls cell growth and differentiation. With MK2 ko mice, MK5 ko mice and double ko mice (both MK2 and MK5), we would be able to identify the specific functions of MK5 and demonstrate if MK5 plays a role on inflammatory response, apoptosis or cell division.

REFERENCE

Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR.(2000) Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Mol Cell.* 6:109-16.

Andoh A, Masuda A, Yamakawa M, Kumazawa Y, Kasajima T.(2000) Absence of interleukin-4 enhances germinal center reaction in secondary immune response. *Immunol Lett.* 73:35-41.

Baeuerle PA, Baltimore D.(1996) NF-kappa B: ten years after. *Cell.* 87:13-20.

Bagrodia S, Derijard B, Davis RJ, Cerione RA.(1995) Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *J Biol Chem.* 270:27995-8.

Behrens A, Sibilia M, Wagner EF. (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat Genet.* 21:326-9.

Bhat NR, Zhang P, Bhat AN.(1999) Cytokine induction of inducible nitric oxide synthase in an oligodendrocyte cell line: role of p38 mitogen-activated protein kinase activation. *J Neurochem.* 72:472-8.

Blanque R, Cottereaux C, Gardner CR.(1997) Effects of SK&F 86002 on cytokine-stimulated IL6 production in cultured neonatal mouse calvaria and SaOS2 osteoblastic cells: the role of prostaglandins and other mechanisms of action. *Drugs Exp Clin Res.* 23:63-70.

Ben-Levy R, Hooper S, Wilson R, Paterson HF, Marshall CJ.(1998) Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. *Curr Biol*. 8:1049-57

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ.(1994) Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell.* 79:59-68.

Cerutti A, Zan H, Schaffer A, Bergsagel L, Harindranath N, Max EE, Casali P.(1998) CD40 ligand and appropriate cytokines induce switching to IgG, IgA, and IgE and coordinated germinal center and plasmacytoid phenotypic differentiation in a human monoclonal IgM+IgD+ B cell line. *J Immunol.* 160:2145-57.

Chang L, Karin M.(2001) Mammalian MAP kinase signalling cascades. *Nature.* 410:37-40

Chen YR, Meyer CF, Tan TH.(1996) Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. *J Biol Chem.* 271:631-4.

Cheng HL, Feldman EL.(1998) Bidirectional regulation of p38 kinase and c-Jun N-terminal protein kinase by insulin-like growth factor-I. *J Biol Chem.* 273:14560-5.

Chung KC, Kim SM, Rhang S, Lau LF, Gomes I, Ahn YS.(2000) Expression of immediate early gene pip92 during anisomycin-induced cell death is mediated by the JNK- and p38-dependent activation of Elk1. *Eur J Biochem.* 267:4676-84.

Clerk A, Michael A, Sugden PH.(1998) Stimulation of the p38 mitogen-activated protein kinase pathway in neonatal rat ventricular myocytes by the G protein-coupled receptor agonists, endothelin-1 and phenylephrine: a role in cardiac myocyte hypertrophy? *J Cell Biol.* 142:523-35.

Cobb MH, Boulton TG, Robbins DJ.(1991) Extracellular signal-regulated kinases: ERKs in progress. *Cell Regul.* 2:965-78.

Constant SL, Bottomly K. (1997) Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol.* 15:297-322.

Corpet F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* 16:10881-90.

Craxton A, Shu G, Graves JD, Saklatvala J, Krebs EG, Clark EA.(1998) p38 MAPK is required for CD40-induced gene expression and proliferation in B lymphocytes. *J Immunol.* 161:3225-36.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC.(1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* 364:229-33.

Cuenda A, Alonso G, Morrice N, Jones M, Meier R, Cohen P, Nebreda AR.(1996) Purification and cDNA cloning of SAPKK3, the major activator of RK/p38 in stress- and cytokine-stimulated monocytes and epithelial cells. *EMBO J.* 15:4156-64.

Cuenda A, Cohen P, Buee-Scherrer V, Goedert M. (1997) Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J.* 16:295-305.

Cuenda A, Dorow DS.(1998) Differential activation of stress-activated protein kinase kinases SKK4/MKK7 and SKK1/MKK4 by the mixed-lineage kinase-2 and mitogen-activated protein kinase kinase (MKK) kinase-1. *Biochem J.* 1998 333 :11-5.

Crawley JB, Rawlinson L, Lali FV, Page TH, Saklatvala J, Foxwell BM. (1997) T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. *J Biol Chem.* 272:15023-7.

de Laszlo SE, Visco D, Agarwal L, Chang L, Chin J, Croft G, Forsyth A, Fletcher D, Frantz B, Hacker C, Hanlon W, Harper C, Kostura M, Li B, Luell S, MacCoss M, Mantlo N, O'Neill Deak M, Clifton AD, Lucocq LM, Alessi DR.(1998) Mitogen- and stress-

activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J.* 17:4426-41.

Davidson SM, Morange M. (2000) Hsp25 and the p38 MAPK pathway are involved in differentiation of cardiomyocytes. *Dev Biol.* 218:146-60.

DeSilva DR, Jones EA, Feeser WS, Manos EJ, Scherle PA.(1997) The p38 mitogen-activated protein kinase pathway in activated and anergic Th1 cells. *Cell Immunol.* 180:116-23.

Diaz-Guerra MJ, Velasco M, Martin-Sanz P, Bosca L.(1996) Evidence for common mechanisms in the transcriptional control of type II nitric oxide synthase in isolated hepatocytes. Requirement of NF-kappaB activation after stimulation with bacterial cellwall products and phorbol esters. *J Biol Chem.* 271:30114-20.

Dong C, Yang DD, Wysk M, Whitmarsh AJ, Davis RJ, Flavell RA.(1998) Defective T cell differentiation in the absence of Jnk1. *Science.* 282:2092-5.

Dong C, Yang DD, Tournier C, Whitmarsh AJ, Xu J, Davis RJ, Flavell RA. (2000) JNK is required for effector T-cell function but not for T-cell activation. *Nature.* 405:91-4.

EA, Orevillo C, Pang M, Parsons J, Rolando A, Sahly Y, Sidler K, O'Keefe SJ, et al.(1998) Pyrroles and other heterocycles as inhibitors of p38 kinase. *Bioorg Med Chem Lett.* 8:2689-94.

Dorion S, Berube J, Huot J, Landry J.(1999) A short lived protein involved in the heat shock sensing mechanism responsible for stress-activated protein kinase 2 (SAPK2/p38) activation. *J Biol Chem.* 274:37591-7.

Doza YN, Cuenda A, Thomas GM, Cohen P, Nebreda AR.(1995) Activation of the MAP kinase homologue RK requires the phosphorylation of Thr-180 and Tyr-182 and both residues are phosphorylated in chemically stressed KB cells. *FEBS Lett.* 364:223-8.

Engelman JA, Lisanti MP, Scherer PE.(1998) Specific inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis. *J Biol Chem.* 273:32111-20.

English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, Cobb MH. (1999) New insights into the control of MAP kinase pathways. *Exp Cell Res.* 253:255-70

Engel K, Schultz H, Martin F, Kotlyarov A, Plath K, Hahn M, Heinemann U, Gaestel M. (1995) Constitutive activation of mitogen-activated protein kinase-activated protein kinase 2 by mutation of phosphorylation sites and an A-helix motif. *J Biol Chem.* 270:27213-21.

Engel K, Ahlers A, Brach MA, Herrmann F, Gaestel M.(1995b) MAPKAP kinase 2 is activated by heat shock and TNF-alpha: in vivo phosphorylation of small heat shock protein results from stimulation of the MAP kinase cascade. J Cell Biochem. 57 321-30.

Engel K, Kotlyarov A, Gaestel M.(1998) Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation. *EMBO J.* 17:3363-71.

Enslen H, Raingeaud J, Davis RJ.(1998) Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J Biol Chem.* 273:1741-8.

Eyers PA, Craxton M, Morrice N, Cohen P, Goedert M.(1998) Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution. *Chem Biol.* 5:321-8.

Eyers PA, van den IJssel P, Quinlan RA, Goedert M, Cohen P.(1999) Use of a drug-resistant mutant of stress-activated protein kinase 2a/p38 to validate the in vivo specificity of SB 203580. *FEBS Lett* 451:191-6.

Fan G, Merritt SE, Kortenjann M, Shaw PE, Holzman LB.(1996) Dual leucine zipper-bearing kinase (DLK) activates p46SAPK and p38mapk but not ERK2. *J Biol Chem.* 271:24788-93.

Foltz IN, Lee JC, Young PR, Schrader JW.(1997) Hemopoietic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. *J Biol Chem.* 272:3296-301.

Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, Saklatvala J. (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* 78:1039-49.

Fukunaga R, Hunter T.(1997) MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.* 16:1921-33.

Furukawa T, Yatsuoka T, Youssef EM, Abe T, Yokoyama T, Fukushige S, Soeda E, Hoshi M, Hayashi Y, Sunamura M, Kobari M, Horii A.(1998) Genomic analysis of DUSP6, a dual specificity MAP kinase phosphatase, in pancreatic cancer. *Cytogenet Cell Genet.* 82:156-9.

Galanis A, Yang SH, Sharrocks AD.(2001) Selective targeting of MAPKs to the ETS domain transcription factor SAP-1. *J Biol Chem.* 276:965-73.

Gallagher TF, Seibel GL, Kassis S, Laydon JT, Blumenthal MJ, Lee JC, Lee D, Boehm JC, Fier-Thompson SM, Abt JW, Soreson ME, Smietana JM, Hall RF, Garigipati RS, Bender PE, Erhard KF, Krog AJ, Hofmann GA, Sheldrake PL, McDonnell PC, Kumar S,

Young PR, Adams JL.(1997) Regulation of stress-induced cytokine production by pyridinylimidazoles; inhibition of CSBP kinase. *Bioorg Med Chem.* 5:49-64.

Ganiatsas S, Kwee L, Fujiwara Y, Perkins A, Ikeda T, Labow MA, Zon LI.(1998) SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and leads to abnormal hepatogenesis. *Proc Natl Acad Sci* 95:6881-6.

Garcia J, Lemercier B, Roman-Roman S, Rawadi G.(1998) A Mycoplasma fermentans-derived synthetic lipopeptide induces AP-1 and NF-kappaB activity and cytokine secretion in macrophages via the activation of mitogen-activated protein kinase *J Biol Chem*. 273:34391-8.

Gause WC, Chen SJ, Greenwald RJ, Halvorson MJ, Lu P, Zhou XD, Morris SC, Lee KP, June CH, Finkelman FD, Urban JF, Abe R. (1997) CD28 dependence of T cell differentiation to IL-4 production varies with the particular type 2 immune response. *J Immunol*. 158:4082-7.

Ge B, Gram H, Di Padova F, Huang B, New L, Ulevitch RJ, Luo Y, Han J.(2002) MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. *Science.* 295:1291-4.

Giroux S, Tremblay M, Bernard D, Cardin-Girard JF, Aubry S, Larouche L, Rousseau S, Huot J, Landry J, Jeannotte L, Charron J. (1999) Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. *Curr Biol.* 9:369-72.

Goedert M, Cuenda A, Craxton M, Jakes R, Cohen P.(1997) Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *EMBO J.* 16:3563-71.

Gratton JP, Morales-Ruiz M, Kureishi Y, Fulton D, Walsh K, Sessa WC.(2001) Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. *J Biol Chem.* 276:30359-65.

Graves JD, Draves KE, Craxton A, Saklatvala J, Krebs EG, Clark EA.(1996) Involvement of stress-activated protein kinase and p38 mitogen-activated protein kinase in mIgM-induced apoptosis of human B lymphocytes. *Proc Natl Acad Sci* 93:13814-8.

Groom LA, Sneddon AA, Alessi DR, Dowd S, Keyse SM.(1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *EMBO J.* 15:3621-32

Guerne PA, Zuraw BL, Vaughan JH, Carson DA, Lotz M.(1989) Synovium as a source of interleukin 6 in vitro. Contribution to local and systemic manifestations of arthritis. *J Clin Invest.* 83:585-92.

Guan KL, Butch E.(1995) Isolation and characterization of a novel dual specific phosphatase, HVH2, which selectively dephosphorylates the mitogen-activated protein kinase. *J Biol Chem.* 270:7197-203.

Guan Z, Buckman SY, Miller BW, Springer LD, Morrison AR.(1998) Interleukin-1beta-induced cyclooxygenase-2 expression requires activation of both c-Jun NH2-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells. J Biol Chem. 273:28670-6.

Gum RJ, McLaughlin MM, Kumar S, Wang Z, Bower MJ, Lee JC, Adams JL, Livi GP, Goldsmith EJ, Young PR.(1998) Acquisition of sensitivity of stress-activated protein kinases to the p38 inhibitor, SB 203580, by alteration of one or more amino acids within the ATP binding pocket. *J Biol Chem.* 273:15605-10.

Han J, Lee JD, Bibbs L, Ulevitch RJ.(1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265:808-11.

Han J, Wang X, Jiang Y, Ulevitch RJ, Lin S.(1997) Identification and characterization of a predominant isoform of human MKK3. *FEBS Lett.* 403:19-22.

Han J, Jiang Y, Li Z, Kravchenko VV, Ulevitch RJ.(1997b) Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature.* 386:296-9.

Hanks SK, Hunter T. (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* 9:576-96.

Hansen TV, Rehfeld JF, Nielsen FC.(1999) Mitogen-activated protein kinase and protein kinase A signaling pathways stimulate cholecystokinin transcription via activation of cyclic adenosine 3',5'-monophosphate response element-binding protein. *Mol Endocrinol.* 13:466-75

Hefner Y, Borsch-Haubold AG, Murakami M, Wilde JI, Pasquet S, Schieltz D, Ghomashchi F, Yates JR 3rd, Armstrong CG, Paterson A, Cohen P, Fukunaga R, Hunter T, Kudo I, Watson SP, Gelb MH.(2000) Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases. *J Biol Chem.* 275:37542-51.

Heidenreich KA, Kummer JL.(1996) Inhibition of p38 mitogen-activated protein kinase by insulin in cultured fetal neurons. *J Biol Chem.*271:9891-4.

Heidenreich O, Neininger A, Schratt G, Zinck R, Cahill MA, Engel K, Kotlyarov A, Kraft R, Kostka S, Gaestel M, Nordheim A.(1999) MAPKAP kinase 2 phosphorylates serum response factor in vitro and in vivo. *J Biol Chem.* 274:14434-43.

Hilberg F, Aguzzi A, Howells N, Wagner EF.(1993) c-jun is essential for normal mouse development and hepatogenesis. *Nature.* 365:179-81.

Hirai S, Katoh M, Terada M, Kyriakis JM, Zon LI, Rana A, Avruch J, Ohno S.(1997) MST/MLK2, a member of the mixed lineage kinase family, directly phosphorylates and activates SEK1, an activator of c-Jun N-terminal kinase/stress-activated protein kinase. *J Biol Chem.* 272:15167-73.

Holtmann H, Winzen R, Holland P, Eickemeier S, Hoffmann E, Wallach D, Malinin NL, Cooper JA, Resch K, Kracht M.(1999) Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Mol Cell Biol.* 19:6742-53.

Hu MC, Wang YP, Mikhail A, Qiu WR, Tan TH.(1999) Murine p38-delta mitogen-activated protein kinase, a developmentally regulated protein kinase that is activated by stress and proinflammatory cytokines. *J Biol Chem.* 274:7095-102.

Huang CK, Zhan L, Ai Y, Jongstra J.(1997) LSP1 is the major substrate for mitogen-activated protein kinase-activated protein kinase 2 in human neutrophils. *J Biol Chem*. 272:17-9.

Huang S, Jiang Y, Li Z, Nishida E, Mathias P, Lin S, Ulevitch RJ, Nemerow GR, Han J.(1997) Apoptosis signaling pathway in T cells is composed of ICE/Ced-3 family proteases and MAP kinase kinase 6b. *Immunity*. 6:739-49.

Huttunen P, Hyypia T, Vihinen P, Nissinen L, Heino J.(1998) Echovirus 1 infection induces both stress- and growth-activated mitogen-activated protein kinase pathways and regulates the transcription of cellular immediate-early genes. Virology. 250:85-93.

Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y.(1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science.* 275:90-4.

Ip YT, Davis RJ.(1998) Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. *Curr Opin Cell Biol*. 10:205-19.

Ishibashi T, Bottaro DP, Chan A, Miki T, Aaronson SA.(1992) Expression cloning of a human dual-specificity phosphatase. *Proc Natl Acad Sci* 89:12170-4.

Ishibashi T, Bottaro DP, Michieli P, Kelley CA, Aaronson SA.(1994)A novel dual specificity phosphatase induced by serum stimulation and heat shock. *J Biol Chem.* 269:29897-902.

Janknecht R, Hunter T.(1997) Convergence of MAP kinase pathways on the ternary complex factor Sap-1a. *EMBO J*. 16:1620-7.

Janknecht R.(2001) Cell type-specific inhibition of the ETS transcription factor ER81 by mitogen-activated protein kinase-activated protein kinase 2. *J Biol Chem.* 276:41856-61.

Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J. (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J Biol Chem*. 271:17920-6.

Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Di Padova F, Ulevitch RJ, Han J.(1997) Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta. *J Biol Chem.* 272:30122-8.

Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med.* 6:41-8.

Juo P, Kuo CJ, Reynolds SE, Konz RF, Raingeaud J, Davis RJ, Biemann HP, Blenis J.(1997) Fas activation of the p38 mitogen-activated protein kinase signalling pathway requires ICE/CED-3 family proteases. *Mol Cell Biol*. 17:24-35.

Kalinski P, Hilkens CM, Wierenga EA, van der Pouw-Kraan TC, van Lier RA, Bos JD, Kapsenberg ML, Snijdewint FG.(1995) Functional maturation of human naive T helper cells in the absence of accessory cells. Generation of IL-4-producing T helper cells does not require exogenous IL-4. *J Immunol*. 1543753-60.

Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee JD.(1997) BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J*. 16:7054-66.

Keyse SM, Emslie EA.(1992) Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature*. 359:644-7.

Kim SJ, Ju JW, Oh CD, Yoon YM, Song WK, Kim JH, Yoo YJ, Bang OS, Kang SS, Chun JS.(2002) ERK-1/2 and p38 Kinase Oppositely Regulate Nitric Oxide-induced Apoptosis of Chondrocytes in Association with p53, Caspase-3, and Differentiation Status. *J Biol Chem.* 277:1332-9.

King CL, Stupi RJ, Craighead N, June CH, Thyphronitis G.(1995) CD28 activation promotes Th2 subset differentiation by human CD4+ cells. *Eur J Immunol*. 25:587-95.

Knauf U, Tschopp C, Gram H.(2001) Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2. *Mol Cell Biol.* 21:5500-11.

Kolodziejczyk SM, Wang L, Balazsi K, DeRepentigny Y, Kothary R, Megeney LA. (1999) MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. *Curr Biol.* 9:1203-6.

Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G.(1993) Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature.* 362:245-8.

Kopf M, Le Gros G, Coyle AJ, Kosco-Vilbois M, Brombacher F.(1995) Immune responses of IL-4, IL-5, IL-6 deficient mice. *Immunol Rev.* 148:45-69.

Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C, Volk HD, Gaestel M. (1999) MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. *Nat Cell Biol.* 1:94-97

Kramer RM, Roberts EF, Um SL, Borsch-Haubold AG, Watson SP, Fisher MJ, Jakubowski JA.(1996) p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J Biol Chem.* 271:27723-9.

Kristof AS, Marks-Konczalik J, Moss J.(2001) Mitogen-activated protein kinases mediate activator protein-1-dependent human inducible nitric-oxide synthase promoter activation. *J Biol Chem.* 276:8445-52.

Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA.(1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron.* 22:667-76.

Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC, Young PR.(1997) Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem Biophys Res Commun.* 235:533-8.

Kumar S, Votta BJ, Rieman DJ, Badger AM, Gowen M, Lee JC. (2001) IL-1- and TNF-induced bone resorption is mediated by p38 mitogen activated protein kinase. *J Cell Physiol.* 187:294-303.

Kummer JL, Rao PK, Heidenreich KA.(1997) Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. *J Biol Chem.* 272:20490-4.

Kwak SP, Dixon JE.(1995) Multiple dual specificity protein tyrosine phosphatases are expressed and regulated differentially in liver cell lines. *J Biol Chem.* 270:1156-60.

Lander HM, Jacovina AT, Davis RJ, Tauras JM.(1996) Differential activation of mitogen-activated protein kinases by nitric oxide-related species. *J Biol Chem.* 271:19705-9.

Lali FV, Hunt AE, Turner SJ, Foxwell BM.(2000) The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. *J Biol Chem.* 275:7395-402.

Latinkic BV, Lau LF.(1994) Transcriptional activation of the immediate early gene pip92 by serum growth factors requires both Ets and CArG-like elements. *J Biol Chem.* 269:23163-70.

Lechner C, Zahalka MA, Giot JF, Moller NP, Ullrich A. (1996) ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. *Proc Natl Acad Sci* 93:4355-9.

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, et al.(1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*. 372:739-46.

Lee JC, Kassis S, Kumar S, Badger A, Adams JL.(1999) p38 mitogen-activated protein kinase inhibitors--mechanisms and therapeutic potentials. *Pharmacol Ther*. 82:389-97.

Li Z, Jiang Y, Ulevitch RJ, Han J.(1996) The primary structure of p38 gamma: a new member of p38 group of MAP kinases. *Biochem Biophys Res Commun*. 228:334-40.

Lim CP, Jain N, Cao X.(1998) Stress-induced immediate-early gene, egr-1, involves activation of p38/JNK1. *Oncogene*. 16:2915-26.

Lisnock J, Tebben A, Frantz B, O'Neill EA, Croft G, O'Keefe SJ, Li B, Hacker C, de Laszlo S, Smith A, Libby B, Liverton N, Hermes J, LoGrasso P.(1998) Molecular basis for p38 protein kinase inhibitor specificity. *Biochemistry*. 37:16573-81.

Liu YJ, Grouard G, de Bouteiller O, Banchereau J. (1996) Follicular dendritic cells and germinal centers. *Int Rev Cytol*. 166:139-79.

Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, Murphy WJ. (1993) Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. Proc Natl Acad Sci U S A. 90:9730-4.

Lu HT, Yang DD, Wysk M, Gatti E, Mellman I, Davis RJ, Flavell RA. (1999) Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. *EMBO J.* 18:1845-57.

Marie C, Roman-Roman S, Rawadi G.(1999) Involvement of mitogen-activated protein kinase pathways in interleukin-8 production by human monocytes and polymorphonuclear cells stimulated with lipopolysaccharide or Mycoplasma fermentans membrane lipoproteins.
Infect Immun. 67:688-93.

Marti F, Krause A, Post NH, Lyddane C, Dupont B, Sadelain M, King PD.(2001) Negative-feedback regulation of CD28 costimulation by a novel mitogen-activated protein kinase phosphatase, MKP6. *J Immunol.* 166:197-206.

Masuda K, Shima H, Kikuchi K, Watanabe Y, Matsuda Y.(2000) Expression and comparative chromosomal mapping of MKP-5 genes DUSP10/Dusp10. *Cytogenet Cell Genet*. 90:71-4.

Masuda K, Shima H, Watanabe M, Kikuchi K.(2001) MKP-7, a novel mitogen-activated protein kinase phosphatase, functions as a shuttle protein. J Biol Chem. 276:39002-11.

Matsumoto T, Turesson I, Book M, Gerwins P, Claesson-Welsh L. (2002). p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. *J. Cell Biol.* 156: 149-60

McLaughlin MM, Kumar S, McDonnell PC, Van Horn S, Lee JC, LiviGP, Young PR.(1996) Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. *J Biol Chem.* 271:8488-92.

McLeish KR, Knall C, Ward RA, Gerwins P, Coxon PY, Klein JB, Johnson GL.(1998) Activation of mitogen-activated protein kinase cascades during priming of human neutrophils by TNF-alpha and GM-CSF. *J Leukoc Biol*. 64:537-45.

Meier R, Rouse J, Cuenda A, Nebreda AR, Cohen P.(1996) Cellular stresses and cytokines activate multiple mitogen-activated-protein kinase kinase homologues in PC12 and KB cells. *Eur J Biochem*. 236:796-805.

Misra-Press A, Rim CS, Yao H, Roberson MS, Stork PJ.(1995) A novel mitogen-activated protein kinase phosphatase. Structure, expression, and regulation. *J Biol Chem.* 270:14587-96.

Miyazawa K, Mori A, Miyata H, Akahane M, Ajisawa Y, Okudaira H.(1998) Regulation of interleukin-1beta-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase. *J Biol Chem.* 273:24832-8.

Molnar A, Theodoras AM, Zon LI, Kyriakis JM. (1997) Cdc42Hs, but not Rac1, inhibits serum-stimulated cell cycle progression at G1/S through a mechanism requiring p38/RK. *J Biol Chem.* 272:13229-35.

Mori A, Kaminuma O, Miyazawa K, Ogawa K, Okudaira H, Akiyama K.(1999) p38 mitogen-activated protein kinase regulates human T cell IL-5 synthesis. *J Immunol*. 163:4763-71.

Morooka T, Nishida E.(1998) Requirement of p38 mitogen-activated protein kinase for neuronal differentiation in PC12 cells. *J Biol Chem.* 273:24285-8.

Moriguchi T, Kuroyanagi N, Yamaguchi K, Gotoh Y, Irie K, Kano T, Shirakabe K, Muro Y, Shibuya H, Matsumoto K, Nishida E, Hagiwara M.(1996) A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J Biol Chem.* 271:13675-9.

Muda M, Boschert U, Dickinson R, Martinou JC, Martinou I, Camps M, Schlegel W, Arkinstall S.(1996) MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. *J Biol Chem.* 271:4319-26.

Muda M, Theodosiou A, Rodrigues N, Boschert U, Camps M, Gillieron C, Davies K, Ashworth A, Arkinstall S. (1996b) The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. *J Biol Chem.* 271:27205-8.

Muda M, Boschert U, Smith A, Antonsson B, Gillieron C, Chabert C, Camps M, Martinou I, Ashworth A, Arkinstall S.(1997) Molecular cloning and functional characterization of a novel mitogen-activated protein kinase phosphatase, MKP-4. *J Biol Chem.* 272:5141-51.

Nagata Y, Moriguchi T, Nishida E, Todokoro K.(1997) Activation of p38 MAP kinase pathway by erythropoietin and interleukin-3. *Blood.* 90:929-34.

Nathan C, Xie QW.(1994) Regulation of biosynthesis of nitric oxide. *J Biol Chem.* 269:13725-8.

Neininger A, Thielemann H, Gaestel M.(2001) FRET-based detection of different conformations of MK2. *EMBO Rep.* 2:703-8.

Neufeld B, Grosse-Wilde A, Hoffmeyer A, Jordan BW, Chen P, Dinev D, Ludwig S, Rapp UR.(2000) Serine/Threonine kinases 3pK and MAPK-activated protein kinase 2 interact with the basic helix-loop-helix transcription factor E47 and repress its transcriptional activity. *J Biol Chem.* 275:20239-42.

New L, Jiang Y, Zhao M, Liu K, Zhu W, Flood LJ, Kato Y, Parry GC, Han J.(1998) PRAK, a novel protein kinase regulated by the p38 MAP kinase. *EMBO J.* 17:3372-84.

New L, Zhao M, Li Y, Bassett WW, Feng Y, Ludwig S, Padova FD, Gram H, Han J.(1999) Cloning and characterization of RLPK, a novel RSK-related protein kinase. *J Biol Chem.* 274:1026-32.

Ni H, Wang XS, Diener K, Yao Z.(1998) MAPKAPK5, a novel mitogen-activated protein kinase (MAPK)-activated protein kinase, is a substrate of the extracellular-regulated kinase (ERK) and p38 kinase. *Biochem Biophys Res Commun*. 243:492-6.

Nick JA, Avdi NJ, Young SK, Knall C, Gerwins P, Johnson GL, Worthen GS.(1997) Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. *J Clin Invest*. 99:975-86.

Niiro H, Otsuka T, Ogami E, Yamaoka K, Nagano S, Akahoshi M, Nakashima H, Arinobu Y, Izuhara K, Niho Y.(1998) MAP kinase pathways as a route for regulatory mechanisms of IL-10 and IL-4 which inhibit COX-2 expression in human monocytes. *Biochem Biophys Res Commun.* 250:200-5

Nishina H, Bachmann M, Oliveira-dos-Santos AJ, Kozieradzki I, Fischer KD, Odermatt B, Wakeham A, Shahinian A, Takimoto H, Bernstein A, Mak TW, Woodgett JR, Ohashi PS, Penninger JM.(1997) Impaired CD28-mediated interleukin 2 production and proliferation in stress kinase SAPK/ERK1 kinase (SEK1)/mitogen-activated protein kinase kinase 4 (MKK4)-deficient T lymphocytes. *J Exp Med.* 186:941-53.

Ono K, Han J.(2000) The p38 signal transduction pathway: activation and function. *Cell Signal.* 12:1-13.

Pages G, Guerin S, Grall D, Bonino F, Smith A, Anjuere F, Auberger P, Pouyssegur. (1999) Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science.* 286:1374-7.

Parker CG, Hunt J, Diener K, McGinley M, Soriano B, Keesler GA, Bray J, Yao Z, Wang XS, Kohno T, Lichenstein HS.(1998) Identification of stathmin as a novel substrate for p38 delta. *Biochem Biophys Res Commun.* 249:791-6.

Pietersma A, Tilly BC, Gaestel M, de Jong N, Lee JC, Koster JF, Sluiter W. (1997) p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem Biophys Res Commun*. 230:44-8

Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC, Saito H.(1996)Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. *Cell.* 86:865-75.

Pugazhenthi S, Boras T, O'Connor D, Meintzer MK, Heidenreich KA, Reusch JE.(1999) Insulin-like growth factor I-mediated activation of the transcription factor cAMP response element-binding protein in PC12 cells. Involvement of p38 mitogen-activated protein kinase-mediated pathway. *J Biol Chem.* 274:2829-37.

Pyne NJ, Pyne S.(1997) Platelet-derived growth factor activates a mammalian Ste20 coupled mitogen-activated protein kinase in airway smooth muscle. *Cell Signal.* 9:311-7.

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ.(1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem.* 270:7420-6.

Ray RP, Schüpbach T.(1996) Intercellular signaling and the polarization of body axes during Drosophila oogenesis. *Genes Dev.* 10:1711-23.

Reimold AM, Grusby MJ, Kosaras B, Fries JW, Mori R, Maniwa S, Clauss IM, Collins T, Sidman RL, Glimcher MJ, Glimcher LH.(1996) Chondrodysplasia and neurological abnormalities in ATF-2-deficient mice. *Nature.* 379:262-5.

Reimold AM, Kim J, Finberg R, Glimcher LH.(2001) Decreased immediate inflammatory gene induction in activating transcription factor-2 mutant mice. *Int Immunol.* 13:241-8.

Rincon M, Whitmarsh A, Yang DD, Weiss L, Derijard B, Jayaraj P, Davis RJ, Flavell RA. (1998) The JNK pathway regulates the In vivo deletion of immature CD4(+)CD8(+) thymocytes. *J Exp Med.* 188:1817-30.

Roberson MS, Zhang T, Li HL, Mulvaney JM.(1999) Activation of the p38 mitogen-activated protein kinase pathway by gonadotropin-releasing hormone. Endocrinology. 140:1310-8.

Rohan PJ, Davis P, Moskaluk CA, Kearns M, Krutzsch H, Siebenlist U, Kelly K.(1993) PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. *Science.* 259:1763-6.

Rolli M, Kotlyarov A, Sakamoto KM, Gaestel M, Neininger A.(1999) Stress-induced stimulation of early growth response gene-1 by p38/stress-activated protein kinase 2 is mediated by a cAMP-responsive promoter element in a MAPKAP kinase 2-independent manner. *J Biol Chem.* 274:19559-64

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, Nebreda AR.(1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell.* 78:1027-37.

Rousseau S, Houle F, Landry J, Huot J. (1997) p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene.* 15:2169-77.

Rutault K, Hazzalin CA, Mahadevan LC.(2001) Combinations of ERK and p38 MAPK inhibitors ablate tumor necrosis factor-alpha (TNF-alpha) mRNA induction. Evidence for selective destabilization of TNF-alpha transcripts. *J Biol Chem.* 276:6666-74

Sabapathy K, Hu Y, Kallunki T, Schreiber M, David JP, Jochum W, Wagner EF, Karin M. (1999) JNK2 is required for efficient T-cell activation and apoptosis but not for normal lymphocyte development. *Curr Biol.* 9:116-25.

Sabapathy K, Jochum W, Hochedlinger K, Chang L, Karin M, Wagner EF.(1999b) Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech Dev.* 89:115-24.

Salmon RA, Foltz IN, Young PR, Schrader JW.(1997) The p38 mitogen-activated protein kinase is activated by ligation of the T or B lymphocyte antigen receptors, Fas or CD40, but suppression of kinase activity does not inhibit apoptosis induced by antigen receptors. *J Immunol.* 159:5309-17.

Schafer PH, Wadsworth SA, Wang L, Siekierka JJ.(1999) p38 alpha mitogen-activated protein kinase is activated by CD28-mediated signaling and is required for IL-4 production by human CD4+CD45RO+ T cells and Th2 effector cells. *J Immunol.* 162:7110-9.

Shalom-Barak T, Quach J, Lotz M.(1998) Interleukin-17-induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-kappaB. *J Biol Chem.* 273:27467-73.

Shapiro L, Puren AJ, Barton HA, Novick D, Peskind RL, Shenkar R, Gu Y, Su MS, Dinarello CA. (1998) Interleukin 18 stimulates HIV type 1 in monocytic cells. *Proc Natl Acad Sci* 95:12550-5.

Schultz H, Engel K, Gaestel M.(1997) PMA-induced activation of the p42/44ERK- and p38RK-MAP kinase cascades in HL-60 cells is PKC dependent but not essential for differentiation to the macrophage-like phenotype. *J Cell Physiol*. 173:310-8.

Sloan SR, Shen CP, McCarrick-Walmsley R, Kadesch T.(1996) Phosphorylation of E47 as a potential determinant of B-cell-specific activity. *Mol Cell Biol.* 16:6900-8.

Smith A, Price C, Cullen M, Muda M, King A, Ozanne B, Arkinstall S, Ashworth A.(1997) Chromosomal localization of three human dual specificity phosphatase genes (DUSP4, DUSP6, and DUSP7). *Genomics.* 42:524-7.

Snow ET.(1992) Metal carcinogenesis: mechanistic implications. *Pharmacol Ther*. 53:31-65.

Stokoe D, Engel K, Campbell DG, Cohen P, Gaestel M.(1992) Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. *FEBS Lett.* 313:307-13.

Stokoe D, Caudwell B, Cohen PT, Cohen P.(1993) The substrate specificity and structure of mitogen-activated protein (MAP) kinase-activated protein kinase-2. *Biochem J.* 296:843-9.

Sugden PH, Clerk A. (1997) Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. *Cell Signal.* 9:337-51.

Sun H, Charles CH, Lau LF, Tonks NK.(1993) MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. *Cell.* 75:487-93.

Sun P, Lou L, Maurer RA.(1996) Regulation of activating transcription factor-1 and the cAMP response element-binding protein by Ca2+/calmodulin-dependent protein kinases type I, II, and IV. *J Biol Chem.* 271:3066-73

Suzanne M, Irie K, Glise B, Agnes F, Mori E, Matsumoto K, Noselli S.(1999) The Drosophila p38 MAPK pathway is required during oogenesis for egg asymmetric development. *Genes Dev.* 13:1464-74.

Suzuki K, Hino M, Kutsuna H, Hato F, Sakamoto C, Takahashi T, Tatsumi N, Kitagawa S.(2001) Selective Activation of p38 Mitogen-Activated Protein Kinase Cascade in Human Neutrophils Stimulated by IL-1beta. *J Immunol.* 167:5940-7

Takenaka K, Moriguchi T, Nishida E. (1998) Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. *Science.* 280:599-602.

Takekawa M, Posas F, Saito H.(1997) A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stress-induced activation of the p38 and JNK pathways. *EMBO J.* 16:4973-82.

Takekawa M, Maeda T, Saito H.(1998) Protein phosphatase 2C alpha inhibits the human stress-responsive p38 and JNK MAPK pathways. *EMBO J.* 17:4744-52.

Tamura K, Sudo T, Senftleben U, Dadak AM, Johnson R, Karin M. (2000) Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. *Cell.* 102:221-31.

Tan Y, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ.(1996) FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2.
EMBO J. 15:4629-42.

Tanoue T, Maeda R, Adachi M, Nishida E. (2001) Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions. *EMBO J.* 20:466-79.

Tanoue T, Yamamoto T, Maeda R, Nishida E.(2001b) A Novel MAPK phosphatase MKP-7 acts preferentially on JNK/SAPK and p38 alpha and beta MAPKs. *J Biol Chem.* 276:26629-39.

Tanoue T, Moriguchi T, Nishida E.(1999) Molecular cloning and characterization of a novel dual specificity phosphatase, MKP-5. *J Biol Chem.* 274:19949-56.

Tew JG, Wu J, Qin D, Helm S, Burton GF, Szakal AK.(1997) Follicular dendritic cells and presentation of antigen and costimulatory signals to B cells. *Immunol Rev.* 156:39-52.

Theodosiou AM, Rodrigues NR, Nesbit MA, Ambrose HJ, Paterson H, McLellan-Arnold E, Boyd Y, Leversha MA, Owen N, Blake DJ, Ashworth A, Davies KE.(1996) A member of the MAP kinase phosphatase gene family in mouse containing a complex trinucleotide repeat in the coding region. *Hum Mol Genet.* 5:675-84.

Thomas G, Haavik J, Cohen P.(1997) Participation of a stress-activated protein kinase cascade in the activation of tyrosine hydroxylase in chromaffin cells. *Eur J Biochem.* 247:1180-9.

Thomson S, Clayton AL, Hazzalin CA, Rose S, Barratt MJ, Mahadevan LC.(1999) The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. *EMBO J.* 18:4779-93.

Tibbles LA, Ing YL, Kiefer F, Chan J, Iscove N, Woodgett JR, Lassam NJ.(1996) MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. *EMBO J.* 15:7026-35.

Tong L, Pav S, White DM, Rogers S, Crane KM, Cywin CL, Brown ML, Pargellis CA.(1997) A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. *Nat Struct Biol.* 4:311-6.

Vajdy M, Kosco-Vilbois MH, Kopf M, Kohler G, Lycke N.(1995) impaired mucosal immune responses in interleukin 4-targeted mice. *J Exp Med.* 18:41-53.

Vancompernolle K, Boonefaes T, Mann M, Fiers W, Grooten J.(2000) Tumor necrosis factor-induced microtubule stabilization mediated by hyperphosphorylated oncoprotein 18 promotes cell death. *J Biol Chem.* 275:33876-82.

Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN.(1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature. 380:75-9.

Wang W, Zhou G, Hu MC, Yao Z, Tan TH.(1997) Activation of the hematopoietic progenitor kinase-1 (HPK1)-dependent, stress-activated c-Jun N-terminal kinase (JNK) pathway by transforming growth factor beta (TGF-beta)-activated kinase (TAK1), a kinase mediator of TGF beta signal transduction. *J Biol Chem.* 272:22771-5.

Wang XS, Diener K, Manthey CL, Wang S, Rosenzweig B, Bray J, Delaney J, Cole CN, Chan-Hui PY, Mantlo N, Lichenstein HS, Zukowski M, Yao Z.(1997) Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. *J Biol Chem.* 272:23668-74.

Wang XZ, Ron D.(1996) Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science.* 272:1347-9.

Wang Y, Huang S, Sah VP, Ross J Jr, Brown JH, Han J, Chien KR.(1998) Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J Biol Chem.* 273:2161-8.

Wang Z, Canagarajah BJ, Boehm JC, Kassisa S, Cobb MH, Young PR, Abdel-Meguid S, Adams JL, Goldsmith EJ.(1998) Structural basis of inhibitor selectivity in MAP kinases. *Structure.* 6:1117-28.

Ward SG, Parry RV, Matthews J, O'Neill L.(1997) A p38 MAP kinase inhibitor SB203580 inhibits CD28-dependent T cell proliferation and IL-2 production. *Biochem Soc Trans.* 25:304S

Waskiewicz AJ, Flynn A, Proud CG, Cooper JA.(1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO J. 16:1909-20.

Webb LM, Feldmann M.(1995) Critical role of CD28/B7 costimulation in the development of human Th2 cytokine-producing cells. *Blood.* 86:3479-86.

Werz O, Klemm J, Samuelsson B, Radmark O.(2000) 5-lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases. *Proc Natl Acad Sci* 97:5261-6.

Whitmarsh AJ, Yang SH, Su MS, Sharrocks AD, Davis RJ.(1997) Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. *Mol Cell Biol.* 17:2360-71.

Wu J, Qin D, Burton GF, Szakal AK, Tew JG. (1996) Follicular dendritic cell-derived antigen and accessory activity in initiation of memory IgG responses in vitro. *J Immunol.* 157:3404-11.

Wu Z, Woodring PJ, Bhakta KS, Tamura K, Wen F, Feramisco JR, Karin M, Wang JY, Puri PL. (2000) p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. *Mol Cell Biol.* 20:3951-64.

Wurgler-Murphy SM, Maeda T, Witten EA, Saito H.(1997) Regulation of the Saccharomyces cerevisiae HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. *Mol Cell Biol.* 17:1289-97.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME.(1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science.* 270:1326-31.

Xie QW, Whisnant R, Nathan C.(1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J Exp Med.* 1993 177:1779-84.

Xie QW, Kashiwabara Y, Nathan C.(1994) Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem.* 269:4705-8.

Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, Rakic P, Flavell RA. (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature.* 389:865-70.

Yang DD, Conze D, Whitmarsh AJ, Barrett T, Davis RJ, Rincón M, Flavell RA.(1998) Differentiation of CD4+ T Cells to Th1 Cells Requires MAP Kinase JNK2 *Immunity* 9: 575-85.

Yang SH, Galanis A, Sharrocks AD.(1999) Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. *Mol Cell Biol.* 19:4028-38.

Young PR, McLaughlin MM, Kumar S, Kassis S, Doyle ML, McNulty D, Gallagher TF, Fisher S, McDonnell PC, Carr SA, Huddleston MJ, Seibel G, Porter TG, Livi GP, Adams JL,
Lee JC.(1997) Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *J Biol Chem.* 272:12116-21.

Yuasa T, Ohno S, Kehrl JH, Kyriakis JM. (1998) Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein associates with a mitogen-activated protein kinase kinase kinase upstream of MKK6 and p38. *J Biol Chem.* 273:22681-92.

Yue TL, Ni J, Romanic AM, Gu JL, Keller P, Wang C, Kumar S, Yu GL, Hart TK, Wang X, Xia Z, DeWolf WE Jr, Feuerstein GZ.(1999) TL1, a novel tumor necrosis factor-like cytokine, induces apoptosis in endothelial cells. Involvement of activation of stress protein kinases (stress-activated protein kinase and p38 mitogen-activated protein kinase) and caspase-3-like protease.*J Biol Chem.* 274:1479-86.

Zhang S, Han J, Sells MA, Chernoff J, Knaus UG, Ulevitch RJ, Bokoch GM.(1995) Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J Biol Chem.* 270:23934-6.

Zetser A, Gredinger E, Bengal E.(1999) p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the Mef2c transcription factor. *J Biol Chem.* 274:5193-200.

Zhang J, Salojin KV, Gao JX, Cameron MJ, Bergerot I, Delovitch TL.(1999) p38 mitogen-activated protein kinase mediates signal integration of TCR/CD28 costimulation in primary murine T cells. *J Immunol.* 162:3819-29.

Zhao M, New L, Kravchenko VV, Kato Y, Gram H, di Padova F, Olson EN, Ulevitch RJ, Han J. (1999) Regulation of the MEF2 family of transcription factors by p38. *Mol Cell Biol.* 19 :21-30.

Zhen X, Wei L, Wu Q, Zhang Y, Chen Q.(2001) Mitogen-activated protein kinase p38 mediates regulation of chondrocyte differentiation by parathyroid hormone. *J Biol Chem.* 276:4879-85.

Declaration

I hereby declare that, all the work presented in this manuscript is my own, carried out solely with the help of the literature and aid cited.

Hannover, April 2002

Yu Shi

Declaration

I hereby declare that, all the results presented in this manuscript have not been published yet till now.

Hannover, May 2002

Yu Shi

CURRICULUM VITAE

Yu Shi

PRESENT ADDRESS

Institut für Physiologische Chemie
Medizinische Hochschule Hannover
Zentrum Biochemie OE 4310
30625 Hannover
Email address: shiyu803@yahoo.com

EDUCATION

M.S.
Master Degree in Science
June 1988
Beijing Normal University, P. R. China
Thesis: The Changes of Ultrastructure of Colon and the Secretion of Gastrointestinal Hormones in the Experimental "Weakness of Spleen" Animals

B.S.
July 1985
Bachelor Degree in Medicine
Nanjing Railway Medical College, P. R. China

RESEARCH BACKGROUND & WORKING EXPERIENCE

1997 ---present
AG: Matthias Gaestel
2001---present
Institut für Physiologische Chemie
Zentrum Biochemie OE 4310
Medizinische Hochschule Hannover
1998---2001
Institut für Pharmazeutische Biologie
Martin-Luther-Universität Halle-Wittenberg
1997---1998
Max-Delbrück-Centrum für Molekulare Medizin, Berlin
In the past four years, I generated and characterized the mitogen-activated protein kinase activated protein kinases 5 (MAPKAPK 5 or MK 5) knockout mice. I found a very interesting phenotype of this gene targeting mice. I found that MK5 affect on some cytokines releasing and plays very important role in immune response. My results also confirmed that MK2 and MK5 are the main downstream protein kinases of p38 MAPK. Once MKs activated, they can phosphorylate some small heat shock proteins or transcriptional factors and influence some gene transcriptions.

1988---1997
Department of Biology,
Beijing Normal University
Working on the combination of western medicine and traditional Chinese medicine.

PERSONAL INFORMATION

Male, healthy, married, Chinese

PUBLICATIONS:

1. Yu Shi and Matthias Gaestel: In the Cellular Garden of Forking Paths: How p38 MAPKs Signal for Downstream Assistance, *Biol Chem*. Vol.383, 1519-1538, 2002
2. Chen Min, Shi Yu, Shi Lingyun, Li Tan and Zhao Ling: Effect of EGF, SS on hCG secretion and hCGß-mRNA expression in early placenta, *Chinese Bio-chemical Journal*, Vol. 11, No. 3, P483-486, 1995
3. Shi Yu, Shi Lingyun, Liang Keshan and Zhang Zhiwen: Stimultaneous detection of 17a-hydroxylase and luteinizing hormone receptor (LHR) mRNA from rat ovary by ribonuclease protection assay, *Progress in Biochemistry and Biophysics*, Vol. 22, No.5, P455-459, 1995
4. Zhang Qiyuan, Shi Yu, Lu Yong, Ma Bing and Gao Xiaobing: The establishment of experimental weakness of spleen (Pi-Xu) animal model of the tree shrew (tupala belangeri chinesis), *Journal of Beijing Normal University (Natural Science),* Vol. 31, No.1, P111-114, 1995
5. Shi Yu, Zhang Qiyuan, Peng Weimin and Fu Xueqing: The ultrastructural and histochemical observations about the effect of „Tanguticum“ on hepatic lesion of mouse induced by galactosamine, *Journal of Beijing Normal University (Natural Science*), Vol. 31, No.2, P271-274, 1995
6. Lu Ming, Shi Yu, Li Weixiong: The influence of tamoxifen and estradiolon c-myc protoongene expression in rat uterus, *Reproduction and Contraception*, Supplement 28, 1994.