C-ABL PROMOTES OSTEOBLAST EXPANSION THROUGH BMP SIGNALING PATHWAYS

KUA HUI YI
(B.Sc (Hons),
University of Manchester)

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

INSTITUTE OF MOLECULAR AND CELL BIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2008

ACKNOWLEDGEMENTS

I wish to express my gratitude and appreciation to my supervisor Dr. Li Baojie, whom without whose guidance and patience this work would have not been possible. I am forever thankful for the advice given over this project, and the opportunities to learn and experiment that make my studentship fulfilling.

I also extend my many thanks and grace:

To my committee advisors Dr. Luo Yan and Dr. Simon Cool, for the insightful discussion that I learnt to enjoy, for the much needed advice and encouragement to help me succeed as a student.

To my collaborators Dr. James Yeh, Guo Ke, Dr. Keith Rogers, Dr. Susan Rogers for their expertise and dedication in getting the excellent bone/histology results for this project. It would not have been possible without their help.

To all my former and current lab colleagues Peter Leong, Zhou Tielin, Ball, Hu Yuanyu, Jenny Chau, Ian Hang In, June Lim, Linna Soh, Grace Khoo, Goh Choon Hong, Aw Bijin, Zeng Li, Naslin, Sherry, Tarika, Jianhe and Liuzhi, for extending your help to me in everyway possible, from experiments to helpful discussions, and also for the fun and laughter. Also, to all my past attachment students that have helped me in every little way.

Last but not least, to my family and friends who have given me undivided support, motivation and encouragement along these years that meant the world to me.

Kua Hui Yi

August, 2008

TABLE OF CONTENTS PAGE

ACKNOWLEDGEMENTS ii

TABLE OF CONTENTS iii

LIST OF FIGURES viii

LIST OF TABLES x

LIST OF ABBREVIATIONS xi

PUBLICATIONS xvi

SUMMARY xvii

Chapter 1 General Introduction

1.1 The c-Abl tyrosine kinase 1

1.1.1 The c-Abl gene, isoforms and structure 2

1.1.2 Abl biology using forward/reverse genetics 4

1.1.3 Abl in transformation and therapeutics: BCR-ABL and STI571 6

1.2 Aging and senescence 9

1.2.1 Organismal aging 9

1.2.2 Cellular senescence 10

1.2.2.1 Replicative senescence versus premature senescence 11

1.2.3 The molecular basis of senescence 12

1.2.3.1 Signaling pathways of senescence 15

1.2.3.2 $p16^{INK4a}$, a biomarker of aging 18

1.2.3.3 Biochemical markers of aging 19

1.2.4 Progeroid syndromes 20

1.3 Bone development & pathophysiology 22

1.3.1 Bone tissue and the process of osteogenesis 22

1.3.2 Bone remodeling 23

		Page
1.3.3	Osteoblast biology	26
	1.3.3.1 Osteoblast differentiation and proliferation	26
	1.3.3.2 Signaling pathways in osteoblasts	27
1.3.4	Disorders of bone remodeling	30
	1.3.4.1 Osteoporosis, a disease of low bone turnover	30
	1.3.4.2 Mouse models of senile osteoporosis	32
1.4	**Bone morphogenetic proteins**	**35**
1.4.1	BMP-Smad pathway	36
1.4.2	Modulators of BMP signaling pathway	41
1.4.3	BMP target genes	44
	1.4.3.1 Id proteins	45
1.5	Thesis rationale	48
Chapter 2	**Materials and Methods**	**50**
2.1	Chemicals and antibodies	50
2.2	Cell culture	51
2.3	Cavarial osteoblasts isolation and culture	51
2.4	Senescence associated-beta-galactosidase (SA-β-Gal) assay	52
2.5	Cell proliferation assays	52
2.6	Infection by retrovirus and selection	53
2.7	Bone marrow extraction	54
2.8	Transient transfection	54
2.9	Western blot	54
2.10	Immunoprecipitation	56
2.11	RNA extraction	56
2.12	RNA extraction of femur/long bone	57
2.13	Reverse Transciption – PCR	58
2.14	Luciferase reporter assay	59
2.15	RNA Interference	59

Page

2.16 Cloning 60
2.17 Site directed mutagenesis 60
2.18 Densitometric analysis 61
2.19 Statistical analysis and data presentation 61

Chapter 3 Premature senescence of *c-Abl*$^{-/-}$ osteoblasts and the up-regulation of p16^{INK4a}, a biomarker of aging 62

3.1 *c-Abl*$^{-/-}$ osteoblasts have decreased proliferation ability and undergoes premature senescence 62
3.2 p16^{INK4a} is up-regulated in *c-Abl*$^{-/-}$ osteoblasts 67
3.3 Increased apoptosis in c-*Abl*$^{-/-}$ osteoblast long bones 68
3.4 Summary 73

Chapter 4 Up-regulation of p16^{INK4a} in *c-Abl*$^{-/-}$ osteoblasts is accompanied by increased ERK1/2 activation and decreased Id1 expression 75

4.1 Role of MAPK pathway in the up-regulation of p16^{INK4a} 76
4.2 An opposing role of Id1 in p16 up-regulation 78
4.3 Summary 80

Chapter 5 c-Abl positively regulates Id1, via the BMP-Smads-1/5/8 signaling pathway 82

5.1 c-Abl is likely to augment Id1 expression via the BMP pathway 82
5.2 c-Abl up-regulates Id1 transcription 83
5.3 c-Abl enhances Smad activation to promote expression of BMP target genes 85
5.4 BMP sustain Id1 expression to promote cell proliferation 88
5.5 Summary 94

Page

Chapter 6 c-Abl acts upstream of Smads-1/5/8 at the BMP receptor level to augment BMP-Smad signaling 96

6.1 c-Abl phosphorylates BMP type I receptors 96
6.2 Serial deletion analysis of BMPR1A reveals the carboxy terminal region is phosphorylated by c-Abl 97
6.3 BMPR1A Y453/467 residues are preferentially phosphorylated by Abl kinases 101
6.4 BMPR1A Y453/457/458/467 mutant affects Smad1 activation, but not Smad1 binding to receptor 108
6.5 Mutant BMPR1A affects receptor complex formation 108
6.6 Summary 112

Chapter 7 A negative role of c-Abl in BMP-induced ERK1/2 activation 114

7.1 Dual phases of ERK activation in c-Abl mutant osteoblasts is dependent on the duration of exposure to BMP2 115
7.2 BMPR1A mutant mimics *c-Abl*$^{-/-}$ osteoblasts in ERK activation 116
7.3 Role of mutant BMPR1A in TAB1-TAK complex formation 118
7.4 Summary 122

Chapter 8 BMP signaling in BCR-ABL and the sustenance of bone marrow progenitor cell population 124

8.1 BCR-Abl potentiates BMP-induced Id1 expression, and can be inhibited by imatinib/STI571 treatment 125
8.2 $p16^{INK4a}$ expression and the ageing of the bone marrow cells 126
8.3 Summary 131

Page

Chapter 9 Discussion and future perspectives 133

Chapter 10 References 145

APPENDICES

LIST OF FIGURES

Fig.	Title	Page
1.1	The structural domains of c-Abl.	5
1.2	The two forms of senescence in mammalian cells.	13
1.3	The two major pathways of senescence, the $p16^{INK4a}$ /Rb and $p19^{ARF}$ /p53 signaling pathways.	17
1.4	The process of bone remodeling in normal bones carried out by osteoclasts and osteoblasts.	25
1.5	The various stages of cell fate commitment to osteoblast differentiation.	28
1.6	The TGFβ superfamily of signaling molecules.	37
1.7	The canonical BMP-Smad pathway.	38
1.8	The basic structures of BMP type I and type II S/T kinase receptors.	39
1.9	Id1 in diffentiation and cell growth.	47
3.1	*c-Abl*$^{-/-}$ osteoblasts do not grow well and senesces prematurely.	64
3.2	Up-regulation of $p16^{INK4a}$ expression in *c-Abl*$^{-/-}$ cells.	69
3.3	Increased apoptosis in c-*Abl*$^{-/-}$ osteoblast long bones.	72
4.1	Enhanced activation of ERK1/2 in *c-Abl*$^{-/-}$ osteoblasts.	77
4.2	Down-regulation of Id1 in *c-Abl*$^{-/-}$ osteoblasts.	79
5.1	c-Abl is required for optimal induction of Id1.	84
5.2	c-Abl regulates Id1 at the level of transcription.	86
5.3	c-Abl regulates the activation of BMP-Smad1/5/8.	89
5.4	BMP2-induced Id1 up-regulation promotes osteoblast proliferation.	92
6.1	Post-translational modification of BMP type I receptors by c-Abl.	98
6.2	Mapping of BMPR1A phosphorylation site by c-Abl via receptor truncations.	103
6.3	Identifying the BMPR1A C-terminal tyrosine residues phosphorylated by c-Abl via mutagenesis.	105
6.4	Smad1 activation is compromised in the mutant BMPR1A.	109
6.5	c-Abl facilitated the interaction between BMPRII and BMPR1A.	111
7.1	c-Abl regulates BMP2-induced ERK activation.	117

Fig.	Title	Page
7.2	c-Abl action on BMPR1A affects ERK activation.	119
7.3	c-Abl phosphorylation of BMPR1A counters TAB1 interaction.	121
8.1	Profile of Id1 gene regulation in an innate activated Abl cell line model.	127
8.2	$p16^{INK4a}$ expression in the bone marrow of aging mice.	130

LIST OF TABLES

Fig.	Title	Page
2.1	List of antibodies used	50
2.2	RT-PCR primers list	58

LIST OF ABBREVIATIONS

AA	amino acid
Abl	abelson
ActR	activin receptor
AKT	thymoma viral proto-oncogene
ALK	activin receptor-like kinase
ALP	alkaline phosphatase
AML	acute myelogenous leukemia
A-MuLV	abelson murine leukemia virus
ARG	Abl related gene
ATM	ataxia-telangiectasia mutated
ATP	adenosine triphosphate
BCR-ABL	breakpoint cluster region -Abl
bHLH	basic helix loop helix
BLAST	Basic Local Alignment Search Tool
BMD	basic multicellular unit
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
BrdU	5'-bromo-2'-deoxy-uridine
c-Abl	cellular-Abelson
CBP	creb binding protein
CC	coiled coil
CDK	cyclin dependent kinase
CDKI	cyclin dependant kinase inhibitor
cDNA	complementary deoxyribonucleic Acid

CFU	colony forming unit
c-Kit	stem cell factor receptor /CD117 cytokine receptor
CML	chronic myelogenous leukemia
Co-Smad	common Smad
DKO	double knockout
DNA	deoxyribonucleic acid
DTT	dithiothreithol
ECM	extracellular matrix
EDTA	ethylenediaminetetra acetic acid
EGF	epidermal growth factor
Egr-1	early growth response protein 1
ERK	extracellular signal-regulated kinase
ES	embryonic stem
FGF	fibroblast growth factor
FL	full length
HA	haemagglutinin (Influenza)
HEPES	4-(2-hydroxyethyl)-1-piperazine-n'2-ethane-sulphonic acid
HMGA	high mobility group A
H_2O_2	hydrogen peroxide
HPV-E6	human papilloma virus – E6 oncoprotein
HSC	hematopoietic stem cell
hTERT	human telomerase reverse transcriptase
Id	Inhibitor of differentiation
IGF	insulin-like growth factor
Ihh	Indian hedgehog

IL-6	interleukin - 6
IP	immuno-precipitation
IRS	insulin receptor substrates
JAK	Janus protein kinase
JNK	c-Jun N-terminal kinase
kb	kilobase
kDa	kilo dalton
KD	kinase dead
LB	luria-bertani medium
LIF	leukemia inhibitory factor
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MDM2	mouse double minute 2
MEF	mouse embryonic fibroblast
MH1	mad homology domain 1
MSC	mesenchymal stem cell
Msx2	msh homeobox 2 transcription factor
Myc	myelocytomatosis oncogene
MyoD	myoblast determination transcription factor
NES	nuclear export signal
NFkB	nuclear factor kappa B
NLS	nuclear localization signal
NRTK	non-receptor tyrosine kinase
OB	osteoblast

OC	osteocalcin
OIS	oncogene-induced senescence
OP	osteopontin
ORF	open reading frame
Osx	osterix
$p16^{INK4a}$	p16 inhibitors of CDK4 A protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDP	pyruvate dehydrogenase phosphatase
Ph +	philadelphia chromosome positive
PI3K	phosphoinositide 3-kinase
PKCδ	protein kinase C delta
PLC-γ1	phospholipase C-gamma 1
PPARγ	peroxisome proliferator-activated receptor gamma
PTH	parathyroid hormone
PYK2	proline rich tyrosine kinase 2
RANKL	receptor activator for nuclear factor kappa B ligand
Ras	rat sarcoma viral oncogene
Rb	retinoblastoma
RNA	ribonucleic acid
ROS	reactive oxygen species
R-Smad	receptor Smad
RT-PCR	reverse transcription- polymerase chain reaction

Runx2	runt-related transcription factor 2
SA-B-Gal	senescence-associated –beta- galactosidase
SAHF	senescence-associated heterochromatic foci
SAMP	senescence-accelerated mouse prone
SAMR	senescence-accelerated mouse resistant
SBE	Smad binding element
SDS	sodium dodecyl sulphate
SERMs	selective estrogen receptor modulators
SH2	src homology domain 2
SHC	src homology 2 domain containing transforming protein
Smad	SMA (*c. elegans*) mothers against decapentaplegic (*drosophila*)
Src	rots sarcoma carcinoma
STAT	signal transducer and activator of transcription
TAB	TAK-1 binding protein
TAK	TGF beta activated kinase
TGFβ	transforming growth factor beta
TK	tyrosine kinase
TNFα	tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
Wnt	Wg (*wingless*) and Int
XIAP	X-linked inhibitor of apoptosis
UV	ultra violet

PUBLICATIONS

Hui-Yi Kua, Yuanyu Hu, Xueying Wang, Jenny F. L. Chau, Ke Guo, Ye-Guang Chen, Yuji Mishina, Sharon Boast, Stephen P. Goff, James Yeh, and Baojie Li (2008). Non-receptor tyrosine kinase c-Abl promotes osteoprogenitor expansion through BMP-activated signaling pathways and $p16^{INK4a}$.
(manuscript in revision)

Wang X, Kua HY, Hu Y, Guo K, Zeng Q, Wu Q, Ng HH, Karsenty G, de Crombrugghe B, Yeh J, Li B (2006). p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling.
J Cell Biol. 2006 Jan 2;172(1):115-25.

SUMMARY

c-Abl is a non-receptor tyrosine kinase involved in various cell processes ranging from cell growth to cellular stress response. Mice carrying the null allele for c-Abl exhibit developmental abnormalities and complex phenotypes. My project attempts to address how c-Abl deficiency leads to the osteoporotic phenotype observed in mutant mice.

c-Abl$^{-/-}$ osteoblasts show a reduction in proliferation potential and undergo premature senescence due to up-regulation of $p16^{INK4a}$, a biomarker of senescence. The $p16^{INK4a}$–mediated osteoblast senescence can be regulated by BMPs, which are major regulators of bone remodeling, through ERK1/2 and Id1 in an opposing fashion. Elevated $p16^{INK4a}$ levels were secondary to the down-regulation of Id1 and enhanced activation of ERKs, both known regulators of $p16^{INK4a}$ expression.

Id1, a known target gene of the BMP pathway, was repressed in *c-Abl*$^{-/-}$ osteoblasts. Further investigation showed that c-Abl could regulate Id1 expression and was required for maximal Id1 induction by BMP2. This premature senescence could be rescued by c-Abl reconstitution or ectopic expression of Id1. c-Abl augments Id1 expression by modulating the activation of Smads1/5/8, direct effectors of the BMP pathway. In addition, the expression of several other BMP target genes was found to be compromised by c-Abl deficiency. Hence, c-Abl played a positive role in BMP signaling and I found the link for c-Abl involvement via the regulation of BMP type I receptors (BMPR IA/IB), upstream activators of Smads. c-Abl was found to phosphorylate BMPR1A, on tyrosine residues Y453/457/458/467 found at the carboxy-terminus of BMPR1A. More importantly, co-immunoprecipitation studies

revealed that receptor complex formation between BMPR1 and BMPRII was compromised without the intact tyrosine residues on BMPR1A, validating the significance of c-Abl action on the BMP pathway.

On the other hand, ERKs are positive regulators of $p16^{INK4a}$ and can be activated by BMPs via the TAB1-TAK1 complex. c-Abl repressed BMP-induced ERK activation over long periods of time by affecting the interaction of TAB1 with BMPR1A, forming the basis behind enhanced MAPK-ERK1/2 activation observed in *c-Abl*$^{-/-}$ osteoblasts. Moreover BMPs, secreted by osteoblasts, not only mediate osteoblast differentiation but also promote osteoprogenitor expansion via sustained Id1 expression, by keeping $p16^{INK4a}$ expression repressed. Thus, through the actions of c-Abl on BMPR1A, c-Abl deficiency shifts BMP signal transduction from Smad1/5/8 to favor MAPK-ERK1/2 activation, resulting in up-regulation of $p16^{INK4a}$. For that reason, c-Abl fine-tuning of $p16^{INK4a}$ expression, in part by BMP signaling, is important for promoting osteoprogenitor cell expansion.

These findings place c-Abl in BMP-mediated signaling pathways and establish its importance in osteoprogenitor expansion, linking osteoprogenitor senescence to senile osteoporosis. *c-Abl*$^{-/-}$ mice may represent a mouse model to study aging related osteopenia.

CHAPTER 1

GENERAL INTRODUCTION

The following literature review provides background information for the results section (Chapter 3 to 8). It begins with an overview of c-Abl, a tyrosine kinase central to this research project. The themes of aging is explored at both the organism and cellular level, as well as in bone homeostasis whereby diseases such as osteoporosis arise from abnormal aging of the bones. Lastly, the BMP pathway, vital to these processes is reviewed in greater detail.

1.1 The c-Abl tyrosine kinase

Tyrosine kinases (TKs) are enzymes that catalyze the transfer of phosphate from ATP to tyrosine residues in polypeptides, and there are 90 TKs and 43 TK-like genes, comprising close to one-fifth of total protein kinases identified in the human genome (Krause and Van Etten, 2005). TKs can be broadly grouped into either receptor TKs or non-receptor TKs (NRTK). c-Abl (cellular-Abelson) is a NRTK, and is closely related to the c-Src and Src family of NRTKs as they share a homologous N-terminal portion. ARG (Abl-related gene, or ABL2) is the only known paralogue of c-Abl (Kruh *et al*., 1990).

c-abl, a proto oncogene; came into discovery only after the isolation of A-MuLV (Abelson murine Leukemia Virus), which induced development of lymphosarcoma in mice (Abelson and Rabstein, 1970). c-Abl was found to be the host protein with kinase activity, while fusions with other viral component such as Gag-Abl (v-Abl)

render its transforming capability. The following subsections will briefly collate structural and functional studies done on c-Abl, and also its involvement in pathophysiology of CML.

1.1.1 The c-Abl gene, isoforms and structure

c-Abl is a 150 kDa protein that is highly conserved and expressed ubiquitously. It is present in most cellular compartments, including the nucleus. In humans, the c-Abl gene gives rise to two forms of mRNA products that are 5- and 6.5- kb in length, denoted type Ia and Ib as a result of alternative splicing from separate promoters found on the Abl gene. The murine versions of c-Abl are denoted as type I and IV, equivalents of human type Ia and Ib respectively. Similarly, two isoforms of ARG; A and B exists (Li, 2006).

Figure 1.1A illustrates the structural domains of the c-Abl Ib protein. The proximal N-terminus at the cap region is important for lipid modification of c-Abl as it contains the consensus sequence for myristoylation, which is not present in type Ia (or I for murine) **(Fig. 1.1B)**. The C_{14} myristoyl fatty acid at the amino terminus is essential for regulating c-Abl activation and is the main difference between the gene variants. Following the cap domain are the SH3 (Src-homology 3), SH2, and kinase domain (PTK), which are common to the Src family kinases. In contrast the large, C-terminal region spanning ~90 kDa is unique to c-Abl. The main sections found here are: a large DNA binding domain, an actin binding domain, three nuclear localization signals (NLS), one single nuclear export signal (NES) (Wen *et al*., 1996, Taagepera *et al*., 1998). The features of the latter two domains confer the special ability of c-Abl to

shuttle between the cytoplasm and nucleus (Van Etten, 1999). Hence, the specific roles of c-Abl can be pre-determined by its localization.

The ability of c-Abl to form fusion proteins with other genes gives rise to isoforms such as v-Abl (viral-Abl), BCR-ABL (Breakpoint cluster-ABL), and Tel-Abl. These gene fusions create oncogenes, as all three possess transformation ability, unlike tightly regulated c-Abl, which cannot transform cells even when overexpressed (Van Etten *et al*., 1989). v-Abl is a result of fusion between Gag viral protein (M-MulV) to the SH2 domain of c-Abl, and can be myristoylated, hence its exclusive localization in the cytoplasm. It possesses potent tyrosine kinase activity that is required for cellular transformation of cells such as myeloid cells and fibroblasts, and can induce pre-B cell leukemia in mice.

BCR-ABL, on the other hand, is a fusion protein between the N-terminal sequences of BCR and almost full-length c-Abl. This fusion makes BCR-Abl constitutively active, unlike c-Abl that is normally held in an inactive form in the absence of stimulation. BCR-Abl is localized in the cytoplasm, with the exception of ectoposide-induced DNA damage that triggers its nuclear translocation (Dierov *et al*., 2004). The various fusions at different points of BCR exons create at least three isoforms that differ in their size: p185, p210, and p230. Differences in BCR-ABL isoforms and the cell it targets is likely to contribute to the type of leukemia formed. In the etiology of specific leukemias, expression of $p210^{BCR\text{-}ABL}$ was correlated with Chronic Myelogenous Leukemia (CML) in over 90% of cases, and estimated 33% of adult acute lymphoblastic leukemia (B-ALL) cases with Ph chromosome. $p185^{BCR\text{-}ABL}$ was

associated with the remaining adult Acute Myelogenous Leukemia cases unrelated with Ph chromosome, AML and lymphoma (Melo, 1996, Deininger *et al*., 2000).

1.1.2 Abl biology using forward/reverse genetics

c-Abl is expressed ubiquitously throughout mouse embryonic development, with higher expression in the thymus (Muller *et al*., 1982, Renshaw *et al*., 1988). In humans, high expression of c-Abl was found in hyaline cartilages, adipocytes and ciliated epithelium of adults, while in fetuses the heaviest expression was observed at the sites of endochondral ossification (O'Neill *et al*., 1997). The physiological functions of c-Abl have been studied mainly with the c-Abl knockout mice. Genetic studies of *c-Abl*$^{-/-}$ mice demonstrated its essential role in development. Mice carrying the null allele for c-Abl are mostly perinatal lethal. And those that survive the first 3 days (<50%) exhibit several developmental abnormalities coupled with complex phenotypes such as shortened-lifespan, infertility, runtedness, thymus and spleen atrophies and osteoporosis (Schwartzberg *et al*., 1991, Tybulewicz *et al*., 1991). Some surviving homozygous mutants will develop megaesophagus and anal prolapse from the third month (Li *et al*., 2000). Through the generation of other c-Abl knockout lines bearing c-Abl deletion or truncation it was found that the carboxy-terminal region was critical for c-Abl function *in vivo* (Li *et al*., 2000).

Mice deficient for c-Abl paralogue Arg are normal. However, mice deficient for both c-Abl and Arg are embryonic lethal at 11 dpc., with defects in neural tube closure and massive apoptosis and hemorrhage in the brains (Koleske *et al*. 1998). This suggests a role for c-Abl and Arg in early embryonic development, with Arg sharing some level of functional redundancy.

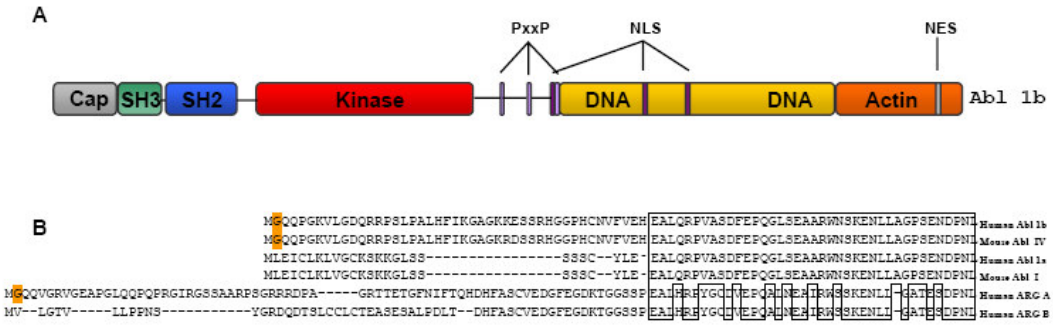


Figure 1.1 The structural domains of c-Abl. (A) Illustration of the domain structures in mammalian c-Abl protein (Abl Ib) and below **(B)** the aligned cap sequences of human Abl Ia, Ib, ARG-A, ARG-B, and mouse c-Abl I and IV. Myristoylation sites are shaded in yellow and the conserved sequences of cap regions are boxed (Li, 2006).

Apart from genetic studies, c-Abl function was extensively studied from the cell biology perspective. Biochemical studies indicate that c-Abl can be activated by various growth factors such as transforming growth factor-β (TGFβ) (Wilkes and Leof, 2006), epidermal growth factor (EGF) (Plattner *et al*, 1999), PDGF through phospholipase C-γ1 (PLC-γ1) (Plattner *et al*., 2003), mainly to promote cell proliferation. It also plays an important role cellular stress response as in the case of promoting apoptosis under DNA damage stress response by activating ATM-p53-p73 pathway (Yuan *et al*., 1999), and in oxidative stress response via protein kinase C (PKCδ) (Li *et al*., 2004). Due to the presence of a huge actin binding domain, c-Abl was also found to regulate cytoskeleton reorganization (reviewed in Pendergast, 2002).

1.1.3 Abl in transformation and therapeutics: BCR-ABL and STI571

Chronic Myelogenous Leukemia (CML) is a clonal myeloproliferative disorder originating from the pluripotential stem cells of the bone marrow. This disease progresses in three stages, starting from the chronic phase, and followed the accelerated phase and lastly, the blast crisis phase. The transition to final two phases often display more aggressive and pleiotropic phenotypes, with a displacement of mature cells by immature blasts (Wong and Witte, 2004). Therefore, treatment of CML in the early stage holds better prognosis, and chances of remission.

The majority of CML patients have the Philadelphia chromosome (Ph+), an abnormal reciprocal translocation between chromosomes 9 (containing c-Abl gene) and 22 (BCR-break point gene) creating partner proteins (Rowley, 1973, Groffen *et al*., 1984, and Heisterkamp *et al*., 1985). This fusion holds a coiled-coil (CC) domain in the

BCR region that mediates oligomerization, which is crucial for its constitutively active kinase activity and remains a target region for therapeutic intervention (reviewed in Krause and Van Etten, 2005).

Imatinib mesylate (Gleevec) was the first and most successful small molecular TK inhibitor tested on CML. It is a 2-phenylaminopyrimidine compound that can specifically inhibit four TKs: Abl, Arg, c-Kit and PDGF receptor (PDGFR) (Druker *et al*., 1996, Okuda *et al*., 2001) and induces complete hematologic and cytogenetic remissions in most chronic phase CML patients (O'Brien *et al*., 2003), with the overall survival rate of 90.8% (Cohen *et al*., 2005).

The elucidation of intramolecular interaction between c-Abl kinase complexed with imatinib using crystallography led to the advanced understanding of the mechanism behind imatinib action (Nagar *et al*., 2003). The latch-clamp model of Abl autoinhibition reveals the conformational changes for c-Abl activation requires removal of myristoyl group, displacement of SH3 domain, along with activation of phosphor-tyrosine sites Y412 and/or Y245. Therefore, how BCR-ABL can maintain constant kinase activation stems from the loss of Cap domain which weakens the inhibitory effect of SH3-SH2 domains and more importantly the oligomerization ability of BCR-CC domain that can facilitate phosphorylation of Y412 (Harrison, 2003, Hantschel and Superti-Furga, 2004).

However, newer approaches to CML therapy is needed as a small percentage of chronic phase patients remain non-responsive to imatinib, and it does not work as well in accelerated and blast stages, with higher incidence of drug resistance in blast crisis patients that received treatment. Moreover, long-term treatment is mandatory as

imatinib acts to repress, but not remove BCR transcripts since it enforces Abl to adopt an inactive confirmation by binding to the conserved ATP-binding region. Long-term toxicity of imatinib is implicated as it can also repress c-Abl functions. Other pharmacological options to improve treatment involve the use of combination therapy (e.g. imatinib with interferon-α or farnesyl transferase inhibitors), small molecule inhibitor nutlin-1 to boost p53 levels. Also, newer designed inhibitors have been tested that are improved versions of imatinib, such as PD173955, AMN107 that inhibits only BCR-ABL, and dual inhibitors of Abl and Src such as AP23464. Other drugs created can also target imatinib-resistant BCR-ABL mutants (reviewed in Li, 2006). The current known pathways that are involved in BCR-ABL pathogenesis will be further discussed in chapter 8.

1.2 Aging and senescence

Aging is a universal, physiological process that marks the finite life-span of a multi-cellular organism, which eventually leads to death. In aging, survival is challenged by deleterious changes that compromise the body's capacity to function efficiently. The history and current understanding of factors involved in the biology of aging are described here, at both the organism and cellular level.

1.2.1 Organismal aging

The typical signs of aging in mice are phenotypes such as reduced life-span, osteopenia, graying hair, reduced weight, skin ulcerations and alopecia (Hasty *et al.*, 2003). In humans, these signs are also prevalent, along with neuronal, retinal degeneration and the increased risk of late-life cancers. Despite the physical similarities, the etiology of aging remains complex, as significant variations in aging phenotypes and individual lifespan have been reported across all species.

Genetics was believed to be the basis for aging. There exist unique sets of pleiotropic genes that function to be beneficial during early life and turn harmful later in age, as natural selection becomes weak, labeled the "antagonistic pleiotropic theory" (Williams, 1957). Less well-received was the theory that aging was programmed, and the accumulation of mutations in genes that confer longevity causes aging. Yet, both theories have not been validated successfully since pleiotropic genes have not been singled out (Partridge and Gems, 2002), and no combination of mutations were found to abolish aging altogether, as seen with genetic manipulations on longevity model, the nematode *C. elegans* (Shaw *et al.*, 1999).

Nevertheless, the "disposable soma theory" could account for the variations observed with aging. The act of natural selection was believed to conserve body's resources to maintain processes such as growth, repair and reproduction. Aging begins when the body's resources are stretched beyond their allocated limit to cater to additional functions such as injury or adaptation to weather. Thus, the exhaustion of resources to maintain optimum body functions is believed to disrupt the maximum lifespan potential of an organism (Kirkwood, 1977 and 2005, Kirkwood and Austad, 2000). Therefore, due to the lack of mechanistic conservation in aging, it remains complicated in understanding the reasons behind aging.

1.2.2 Cellular senescence

Cellular or replicative senescence is an *in vitro* phenomenon that occurs to cells in culture. Unlike tumor cells that can proliferate indefinitely, normal cultured cells were observed to possess a finite lifespan, or doubling time (Hayflick and Moorhead, 1961). The process of replicative senescence is a fundamental feature of somatic cells, and is characterized by cells undergoing permanent growth arrest at G1 phase (Campisi, 1997). Senescent cells adopt a typical physical appearance of a large, flattened morphology. More striking are the biochemical changes that distinguish a senescent cell from a pre-senescent cell. Senescent cells cease to divide but remain metabolically active. Apart from the irreversible block to cell proliferation, changes to expression of different sets of transcription factors, the increased resistance to apoptosis (Wang, 1995) and the secretion of various cytokines and matrix proteins makes a senescent cell alter its normal differentiated functions (Shelton *et al*., 1999, Itahana *et al*., 2004). With the knowledge obtained on senescence mostly from cell culture studies, an important caveat to be questioned is whether senescence takes

place *in vivo*. Campisi addressed this issue and strongly suggested that cells do undergo replicative senescence *in viv*o to contribute to aging, via several lines of evidence that included studies of cells passaged in intact animals (reviewed in Campisi, 1996 and 2001).

The outcome of senescence can be a two-edged sword. Senescence remains an effective barrier against neoplastic transformation, linking cancer and aging as a tumor suppressive mechanism. Yet in contradiction, the presence of senescent cells can accumulate in aged tissues and disrupt tissue integrity and normal function through their altered actions such as the secretion of degradatory matrix proteins (Dimri *et al*., 1995, Millis *et al*., 1992). This in turn may promote cancer progression (Campisi, 2005).

1.2.2.1 Replicative senescence versus premature senescence

Senescence can be categorized into two types, replicative senescence which is attributed by telomerase (hence intrinsic), and non-replicative/premature senescence which occurs by telomere-independent mechanisms (extrinsic). This classification is important in distinguishing the different mechanisms of senescence that occurs between humans and mice.

Intrinsic senescence relies on the expression of telomerase. The telomerase enzyme synthesizes telomeres, which function as a "cap" to keep chromosome length constant. To protect cells against genomic instability and cancer, it is believed senescence is triggered when cells acquire very short telomeres (Kim *et al*., 2002). However, only human germ cells and certain stem cells express telomerase, therefore most human somatic cells suffer from telomere attrition after each round of DNA

replication and become vulnerable to senescence. This cell-counting mechanism employed by human cells does not exist in mice as laboratory mice have been shown to possess extremely long telomeres (40-60 kb, in contrast with 5-15 kb in humans) (reviewed in Itahana *et al*., 2004). Hence, the senescence of murine cells is most likely to be induced by extrinsic or environmental factors. **Figure 1.2** summarizes the two classes of senescence and the causative agents involved.

1.2.3 The molecular basis of senescence

Many intrinsic and extrinsic factors have been implicated to trigger senescence and different hypotheses were proposed along these lines. However, little is done to unify these theories as they remain rather unconnected, while it is generally believed that each of these causes work in combination to contribute to aging as a whole.

One key intrinsic factor that affects the rate of aging is progressive telomere attrition that acts as a system to 'count' cell divisions and a potential tumor-protection mechanism (Wright and Shay, 2001). Yet, the differences in mouse and human telomere lengths demonstrate the species-specific disparity in regulating senescence. The increased levels of oxidative stress was found to be able to accelerate telomere loss (von Zglinicki, 2002), indicating innate and extrinsic factors may share similarities in signaling mechanisms.

Genome maintenance is another important factor. Damage to DNA triggers sensors of DNA double strand breaks and leads to the recruitment of DNA repair foci to the site of damage. Defects in DNA repair systems increase the risk of somatic cell mutations, and thus senescence is induced. Therefore, the capacity for DNA repair is another

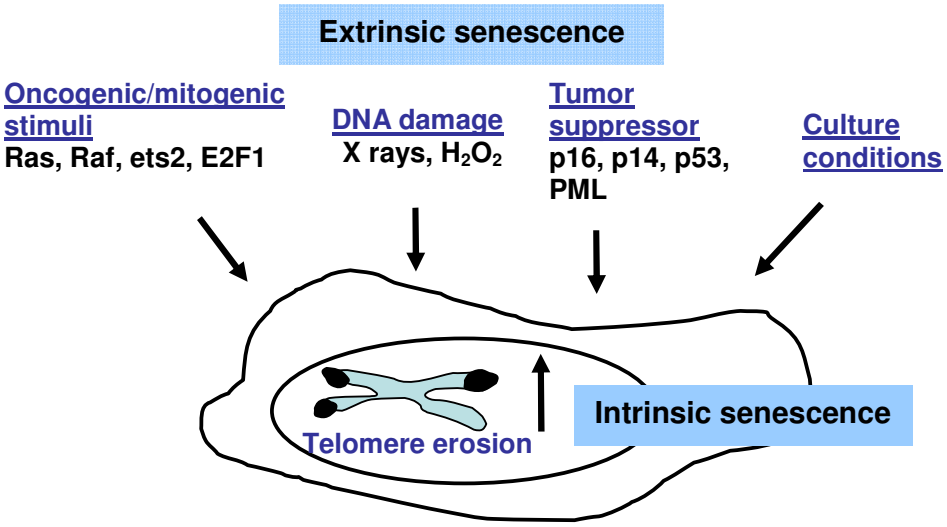


Figure 1.2. The two forms of senescence in mammalian cells. Extrinsic senescence (telomere independent) covers cell culture conditions, DNA damage by radiation or chemicals, oncogenic or mitogenic stimuli and exogenously overexpressed tumor suppressors such as $p16^{INK4a}$. Intrinsic senescence occurs within the cell nucleus, a result of telomere shortening.

important determinant in the rate of aging (Kirkwood, 2005), and human models for this form of aging are discussed in section 1.2.4.

A major extrinsic factor for inducing senescence is the exposure of cells to cellular stresses. STASIS (stress or aberrant signaling-induced senescence) forms the basis of the numerous acute and chronic stress signals that confronts a pre-senescent cell (Shay and Roninson, 2004). Oxidative stress, in particular is common in cell culture conditions where higher physiological concentrations of O_2 and the production of free radicals ROS (reactive oxygen species) can significantly accelerate senescence (Lundberg *et al*., 2000). For example, mild hyperoxia culture conditions could shorten a cell's replicative lifespan and induce accelerated telomere shortening (von Zglinicki *et al*, 1995), while short term serum starvation in cells induced quiescence, and long term serum deprivation triggered senescence. The free radical theory of senescence may be valid in organismal aging as well, since $p66^{SHC-/-}$ mice were less sensitive to the toxic effects of ROS, and lived 30% longer than its wild type counterparts (Migliaccio *et al*., 1999).

The relationship shared by oncogenes and senescence has been well established. Oncogene-induced senescence (OIS) describes the ability of oncogenes such as Myc, E1A or Ras to activate genes ($p16^{INK4a}$ and $p19^{ARF}$) required for cell cycle arrest and induce senescence, when ectopically expressed (Serrano *et al*., 1997, Palmero *et al*., 1998). OIS operate as a bona fide barrier to tumorigenesis *in vivo* (reviewed in Narita and Lowe, 2005), as the escape from senescence or immortality is important for malignant transformation to occur. In this case, senescence can be bypassed by introducing viral oncogenes (e.g. HPV-E6), that targets tumor suppressors p53 and

Rb, both of which must be functionally inactivated in order for human cells to circumvent senescence (Shay *et al*., 1991). Nevertheless, the mechanism of telomere shortening (replicative senescence) appears to be dispensible for ras-induced senescence, since prior ectopic expression of hTERT allowed fibroblasts to escape from replicative, but not ras-induced senescence (Wei and Sedivy, 1999). Furthermore, the immortalization of epithelial cells required additional mechanisms such as epigenetic modifications ($p16^{INK4a}$ methylation) as the expression of telomerase per se is insufficient to bypass senescence (Wright and Shay, 2001). Hence, it appears different senescence mechanisms are employed by cells and this may vary among species, cell type, or in different cell context.

1.2.3.1 Signaling pathways of senescence

Despite the diverse stimuli in inducing a senescent state, only two signaling pathways have been shown to be responsible. Crucial to these pathways are the activation of two key tumor suppressor genes p53 and pRb (retinoblastoma) and their effectors cyclin-dependent kinase inhibitors (CDKIs) $p19^{ARF}$ and $p16^{INK4a}$ respectively, as illustrated in **Figure 1.3**.

In the p53 pathway, $p19^{ARF}$ stabilizes p53 by interfering with its negative regulator MDM2. In turn, $p21^{CIP1}$ gets induced by p53, to promote cell cycle arrest since $p21^{CIP1}$ inhibit cyclin E and A-dependent kinase complexes. The activation of $p19^{ARF}$ /p53 pathway is associated with telomere dysfunction, oncogene ras activation and DNA damage. p53 is vital to DNA damage response signaling pathways (Wahl and Carr, 2001) and it is believed that the shortening of telomeres resemble damaged DNA, thus triggering a p53-mediated response. The effects of p53 activation in senescence may well be an indirect response to the many pathways it regulates. In ras-

induced senescence, p53 activation was postulated to be caused by the DNA-damaging effects of ROS production, created from ras-induced MAPK signaling (reviewed in Campisi, 2005). Unlike Rb-mediated senescence, p53-mediated senescence is reversible, as inactivating p53 in replicative senescent human cells can completely reverse the senescent growth arrest phenotype (Gire and Wynford-Thomas, 1998).

In the Rb pathway, $p16^{INK4a}$ is the key mediator in controlling cell cycle arrest by inhibiting cyclin-dependent kinases, which consist of a catalytic subunit (cdk) and a regulatory subunit (cyclin). The steps of Rb activation begins with $p16^{INK4a}$ induction upon stress stimuli, which in turn bind to cdk4/6 to inactivate cdk4/6-cyclinD complexes via inhibition of ATP binding and thus displacing cyclinD. Consequently the Rb proteins remain hypophosphorylated, leading to G1 arrest, as cdk4/6-cyclinD complexes are required for Rb phosphorylation during G1-S transition (Serrano *et al*, 1993). This inactivation of Rb also lifts its repression of E2F transcription factors, which are responsible for promoting cell proliferation (Stevaux and Dyson, 2002). $p16^{INK4a}$ activation comes mainly from conditions of cellular stress, such as genotoxic and oxidative stress, as well as ras overexpression. The activation of this pathway is irreversible, as once pRb is engaged by $p16^{INK4a}$, the senescence-mediated growth arrest cannot be reversed by subsequent p53 inactivation, silencing of $p16^{INK4a}$, or inactivation of pRb (Beausejour *et al*., 2003). Therefore the $p16^{INK4a}$/Rb pathway may function as a final barrier to cell proliferation, in situations where senescence can be overcome by p53 loss or inactivation (Campisi, 2005).

The activation of either pathway appears to depend on the type and species of origin in cell. In mice, MEFs depend on $p19^{ARF}$ for ras-induced senescence, whereas

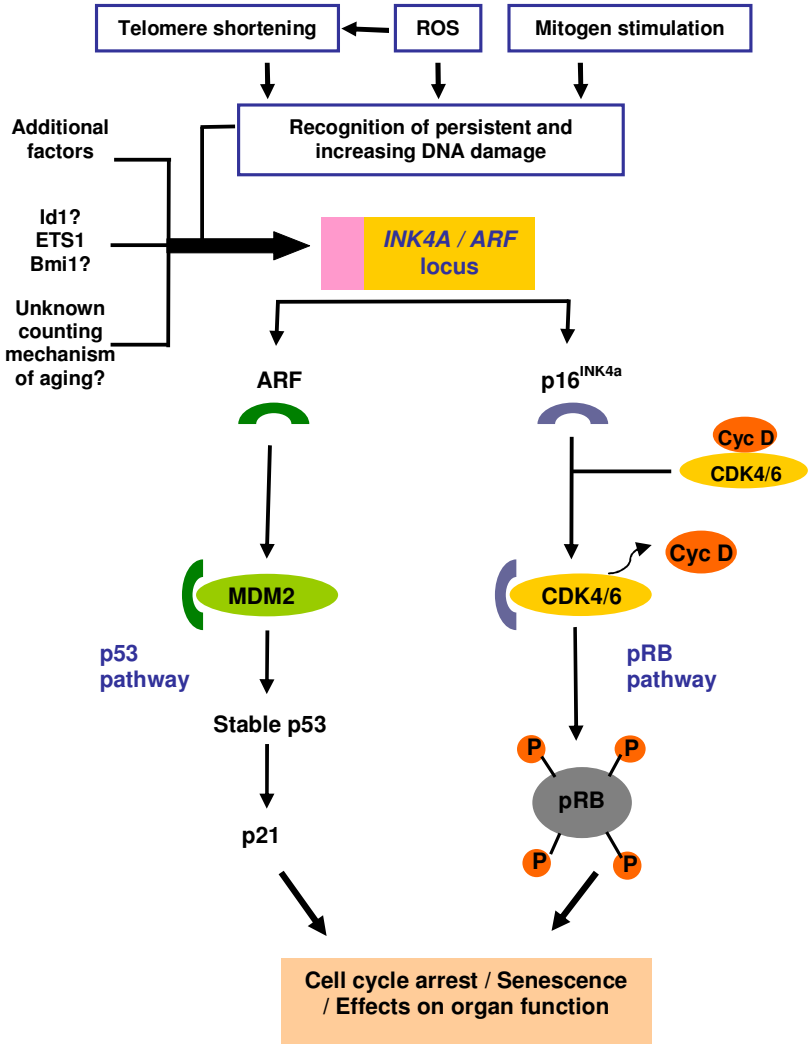


Fig 1.3. The two major pathways of senescence, the $p16^{INK4a}$ /Rb and $p19^{ARF}$ /p53 signaling pathways. Activation by various stimuli, and the key molecules involved in these pathways are illustrated here.

$p16^{INK4a}$ appears to be essential for senescence of human cells since fibroblasts carrying inactivating mutations of $p16^{INK4a}$ can be resistant to ras-induced senescence (Carnero *et al*., 2000, Huot *et al*., 2002). The $p16^{INK4a}$ /Rb pathway was also found to be important for melanocyte senescence, melanoma progression (Bennett, 2003). These pathways may cooperate to regulate senescence, as seen with Pak4 bridging p16/p19 pathways through MAPK-ERK (Cammarano *et al*., 2005), while the inactivation of both pathways can bypass senescence and trigger cell transformation (Hara *et al*., 1991).

1.2.3.2 $p16^{INK4a}$, a biomarker of aging

The INK4 family of proteins regulates cell cycle progression by binding and inhibiting cdks. $p16^{INK4a}$ belongs to this family that also includes $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d/ARF}$. $p16^{INK4a}$ and $p19^{ARF}$ are alternative splice variants from the INK4a locus, and therefore they are not isoforms and share no homology in amino acid sequence (Kim and Sharpless, 2006). Three $p16^{INK4a}$ variants have been found to exist in senescent human fibroblasts (Weebadda *et al*., 2005).

$p16^{INK4a}$ arrest cells at the G1 phase of cell cycle, and the induction of $p16^{INK4a}$ in normal proliferating cells is a relatively rare event (Serrano *et al*, 1993). Instead, $p16^{INK4a}$ expression is normally silenced by methylation in cultured cells as well as *in vivo* (Holst *et al*., 2003). $p16^{INK4a}$ have been established to be a true biomarker of mammalian aging, as its *in vivo* expression correlates with the aging of mice (Krishnamurthy *et al*., 2004). Moreover, the increasing $p16^{INK4a}$ gene dosage (to lesser extent with $p19^{ARF}$) results in normal aging and resistance to cancer (Matheu *et al*.,

2004). More recently, $p16^{INK4a}$ was found to promote the age-dependent decrease in proliferation and self-renewal capacity of progenitor cells in different organs such as brain, bone marrow and pancreatic islets (Molofsky *et al*., 2006, Janzen *et al*., 2006 and Krishnamurthy *et al*., 2006).

Among cdk inhibitors, $p16^{INK4a}$ is the only known bona fide tumor suppressor, since $p16^{INK4a}$ function, or its downstream mediators is frequently de-regulated in many types of human cancers (Okamoto *et al*., 1994). Therefore, $p16^{INK4a}$ and the $p16^{INK4a}$ – pRb pathway remains a molecular link between cellular senescence and tumor suppression (Ohtani *et al*., 2004).

Some progress has been made in understanding the regulation and kinetics of $p16^{INK4a}$. The stable expression of $p16^{INK4a}$ during replicative senescence was a result of decreased $p16^{INK4a}$ mRNA turnover, mediated by a mRNA decay protein AUF1 (Wang *et al*., 2005). $p16^{INK4a}$ was also demonstrated to participate in epigenetic regulation, as high-mobility group A (HMGA) proteins cooperate with $p16^{INK4a}$ to promote senescence-associated heterochromatin foci formation, thus stabilize senescence by silencing E2F target genes (Narita *et al*., 2006).

1.2.3.3 Biochemical markers of aging

To date, current research employs a limited set of candidate markers to define and measure the degree of senescence. Key genes are cell cycle inhibitors $p16^{INK4a}$ and $p19^{ARF}$ (ARF), where their mRNA expression markedly increases with aging. The expression profile of $p16^{INK4a}$ often correlates with the up-regulation of enzyme SA-β-Gal (Senescence-associated-β-Galactosidase), which remains the gold standard for detecting senescence *in vitro and in vivo* (Dimri *et al*., 1995). Interestingly, the

discovery of senescence-associated heterochromatic foci (SAHF), which accumulates in senescent human fibroblasts, may serve as a potential marker. The formation of SAHF was found to correlate with stable repression of E2F target genes, validating the existence of an intact p16-Rb mediated senescent pathway (Narita *et al*., 2003).

1.2.4 Progeroid syndromes

Does senescence reflect aging in humans? Although the features and causative agents of aging vary widely among species, some universal aging mechanisms have been identified, such as alterations to insulin-IGF axis. Yet, mechanisms primary to aging in humans may not necessarily have counterparts in every metazoan (Kipling *et al*., 2004). The study of aging in humans are modeled on progeroid (premature aging-like) syndromes, which are human genetic diseases with outcomes that mimic aging phenotypes (Martin, 1985). However, most progeroid syndromes manifest only partial aging phenotypes, which compound the fear that these syndromes may have resulted from mechanisms postulated to play only causal roles in normal aging (Hasty *et al*., 2003).

Genomic instability remains a fundamental problem as defects in genome maintenance can greatly compromise survival and accelerate aging. In humans, Werner syndrome (WS) displays severe age-related diseases such as type II diabetes, osteoporosis and calcification of cardiac valves, arising from DNA helicase (RecQ) mutation. The ataxia telangiectasia (ATM) syndrome, which exhibits immunodeficiency and osteopenia (Hishiya *et al*., 2005), arises from defects in repair of double strand breaks (reviewed in Lombard *et al*., 2005). Hutchinson-Gilford progeria (HGP), shows distinct tissue specific features, due to mutations in lamin A/C

(Goldman *et al*., 2004). A common ground for comparative studies between humans and mice have been achieved by crossing the Werner gene (*wrn*) to telomerase-null (*terc*$^{-/-}$) mice, in the attempt to humanize the telomere length in mice (Chang *et al*., 2004).

1.3 Bone development & pathophysiology

The physiological functions of bone serve not only as a support system for our internal organs, but also for body movement, body resistance and adaptation to changing mechanical stress/loading. Other essential roles for bone lie in the regulating homeostasis of ions; in particularly calcium, as well as providing the site for hematopoiesis to take place. The biology of bone and disease is reviewed in following sub-headings below.

1.3.1 Bone tissue and the process of osteogenesis

The skeletal system consists mainly of bone tissue, a specialized form of connective tissue with its extracellular matrix (ECM) components calcified. Mineralization confers immense rigidity and tensile strength, characteristics of bone. The average compact bone holds 30% organic matrix and 70% salts in which collagen fibers fill the organic matrix and the gradual deposition of inorganic bone salts (mostly calcium and phosphates which forms hydroxyapatite crystals) increases its compressional strength, along with tensile strength, to create a material reminiscent of reinforced concrete (Guyton and Hall, 1996). Anatomically, bone is comprised of 2 different types of structures, namely the compact bone (cortical bone), which is the outer hard shell that forms the shaft of long bones, and spongy bone (trabecular/cancellous bone), which consists of a lattice-like network of bony spicules with interconnecting spaces filled with bone marrow cells (Burkitt *et al*., 1999).

During development, immature woven bone can be found in a developing embryo, up to childhood before 14 years of age, and temporarily in the regions of bone undergoing repair. Over time, secondary bone takes over to form well organized

lamellar bone, which contains compact and cancellous bone, forming the mature skeleton. The process of endochondral ossification takes place in all long bones. Unlike intramembraneous bone formation (which involves direct bone formation within primitive connective tissue), this process requires the development of a cartilage platform prior to bone formation, and an intricate network of several cell types. Long bone growth involves expansion of bones in diameter and length. The increase in thickness of cortical bone shaft (diaphysis region) can be achieved by bone deposition on the periosteal surface, and resorption at the endosteal surface that begins from the center region of bone and creates a hollow bone marrow cavity. On the other hand, an increase of bone length takes place primarily at the ends of long bones, termed epiphyseal plate region (growth plate). It is the fusion of epiphyses which results in the loss of growth plates and hence cessation of bone growth at the end of adolescence (www.techion.ac.il).

At the cellular level, 3 major cell lineages originating from mesenchymal stem cells critical to osteogenesis are osteoblasts, osteoclasts and chondrocytes. Endochondral ossification of long bones begins with condensation of mesenchymal cells to form chondrocytes (Kronenberg, 2003), laying down a hyaline cartilage template which then directs the differentiation of osteoblasts to form mature bone (Russell *et al*, 2006). Osteoclasts act to remove bone, while osteoblasts function to form new bone.

1.3.2 Bone remodeling

As progression of early bone modeling to formation of mature skeleton is completed, the complicated process of bone remodeling begins. Continuous bone remodeling takes place so as to maintain bone homeostasis, bone architecture and buffer

circulating plasma calcium in the body. Bone remodeling involves creating new bone tissue in place of old bone, and these require the orchestrated actions of osteoblasts and osteoclasts, to form a basic multicellular unit (BMU).

A cycle of BMU action revolves around 3 consecutive phases: bone resorption, reversal and formation. Osteoclasts, multinucleate cells derived from the monocytic/macrophage lineage, resorb bone upon migration to the bone surface, leaving open 'trenches' or cavities that are 20-40μm deep in cortical bones. This resorption stage is then followed by a reversal phase with mononuclear cells appearing on bone surface, to prepare the surface for new bone by signaling for the maturation of osteoblast progenitors and subsequently recruiting osteoblasts to the site to lay down new bone till the resorbed site is fully covered, completing the final phase of the cycle. Flattened lining cells then occupy the site until a new remodeling cycle is initiated. This process is illustrated in **Figure 1.4**.

Hence, the actions of osteoblasts and osteoclasts are closely coupled to each other. Bone turnover occurs more actively in trabecular bones, which form 20% of skeletal mass but holds 80% of total bone surface due to the larger surface to volume ratio. Nevertheless, up to 2-5% of cortical bones, and about 25% of trabecular bones are replaced annually during adulthood (Hadjidakis and Androulakis, 2006, Manolagas and Jilka, 1995). Bone remodeling is regulated by a numerous factors, which range from secondary/systemic influence via the endocrine system (such as parathyroid hormone (PTH) and estrogens), to local regulation in the bone microenvironment via the release of cytokines that regulate osteclasts (osteoprotegerin-OPG), or osteoblasts (interleukin 6- IL6) (reviewed in Hadjidakis and Androulakis, 2006).

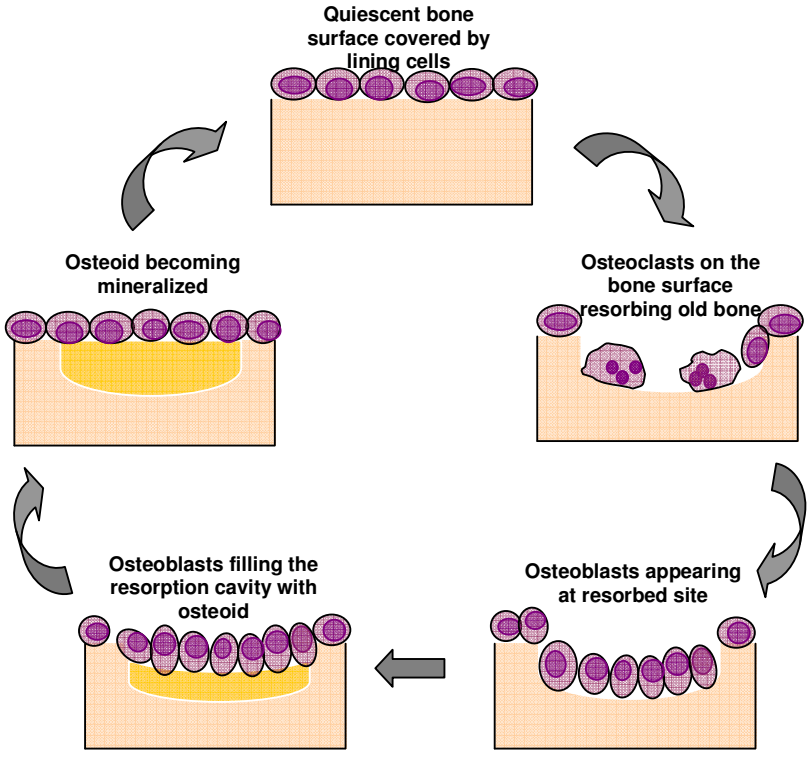


Figure 1.4. The process of bone remodeling in normal bones carried out by osteoclasts and osteoblasts.

1.3.3 Osteoblast biology

Osteoblasts form new bone by first synthesizing complex extracellular matrix including collagen fibres (termed osteoid), which is then subsequently mineralized. Osteoblasts are found in clusters, lining the layer of bone matrix that they are producing on the bone surface. When its matrix secreting period ceases, 15% of mature osteoblasts become entrapped in the new bone matrix and differentiate into osteocytes, while some remain on the bone surface to form flat lining cells (Hadjidakis and Androulakis, 2006). Such functions require osteoblasts to be rich in the enzyme alkaline phosphatase that is needed for the formation of the mineral deposits in the matrix. Due to the metabolic requirements of this cell type, it is also morphologically a large cell filled with abundant basophilic cytoplasm that holds a huge Golgi apparatus, a pale stained nucleus but prominent nucleolus, features that reflect a high rate of protein and proteoglycan synthesis (Burkitt *et al*., 1999).

1.3.3.1 Osteoblast differentiation and proliferation

Cells of the osteoblast lineage originate from stromal cells of bone marrow, where mesenchymal stem cells reside. The pluripotency of these stem cells can give rise to not only osteoblasts, but also adipocytes, chondrocytes, myoblasts or fibroblasts (Bianco *et al* 2001). Fibroblast colony-forming unit (CFU-F) remains the earliest cell to commit to the osteoblast lineage (Manolagas and Jilka, 1995). The commitment of precursor cells to the osteoblastic fate involves the complicated coordination of several cytokines and growth factors, and timed expression of osteoblast specific transcription factors. **Figure 1.5** illustrates the stages of osteoblast differentiation, along with the biochemical markers that are expressed by osteoblasts at different

phases of differentiation. The early steps of cell commitment to preosteoblast remains vague, but several factors have been singled out in driving proliferation and expansion of precursor osteoblasts, and they include bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF), insulin-like growth factors I and II (IGF-I and IGF-II), platelet-derived growth factor (PDGF) and transforming growth factors-β1 and β2 (Mundy *et al*., 2001, Chen *et al*, 2004), though most of these factors act to exert similar effects on mesenchymal cells in general. Moreover, the differentiation program of osteoblast is dependent on the expression of master genes Runx2 (or core binding factor 1/cbfa1), and osterix (osx). Through genetic studies it was found that both transcription factors are essential in bone formation as *Runx2* null mice form a skeleton made entirely of cartilage and not normal bones (Ducy *et al*., 1997, Komori *et al*., 1997), while $Osx^{-/-}$ mice do not develop bones. It was believed that osterix worked downstream of Runx2 as $Runx2^{-/-}$ mice do not express osterix at all, while Runx2 was expressed in $Osx^{-/-}$ mice despite the lack of bone formation(Nakashima *et al*., 2002). Other transcription factors than influence osteoblast differentiation *in vivo* include the homeobox proteins Dlx5, Dlx6 and Msx2 (reviewed in Ducy *et al*., 2000). More work remains to be done on uncovering the regulation of osteoblast differentiation, in comparison to the extensive research and knowledge obtained about osteoclast biology.

1.3.3.2 Signaling pathways in osteoblasts

The signal transduction pathways that occur in osteoblasts can be traced from the substances that they secrete. BMP-mediated signaling, for example, is of utmost

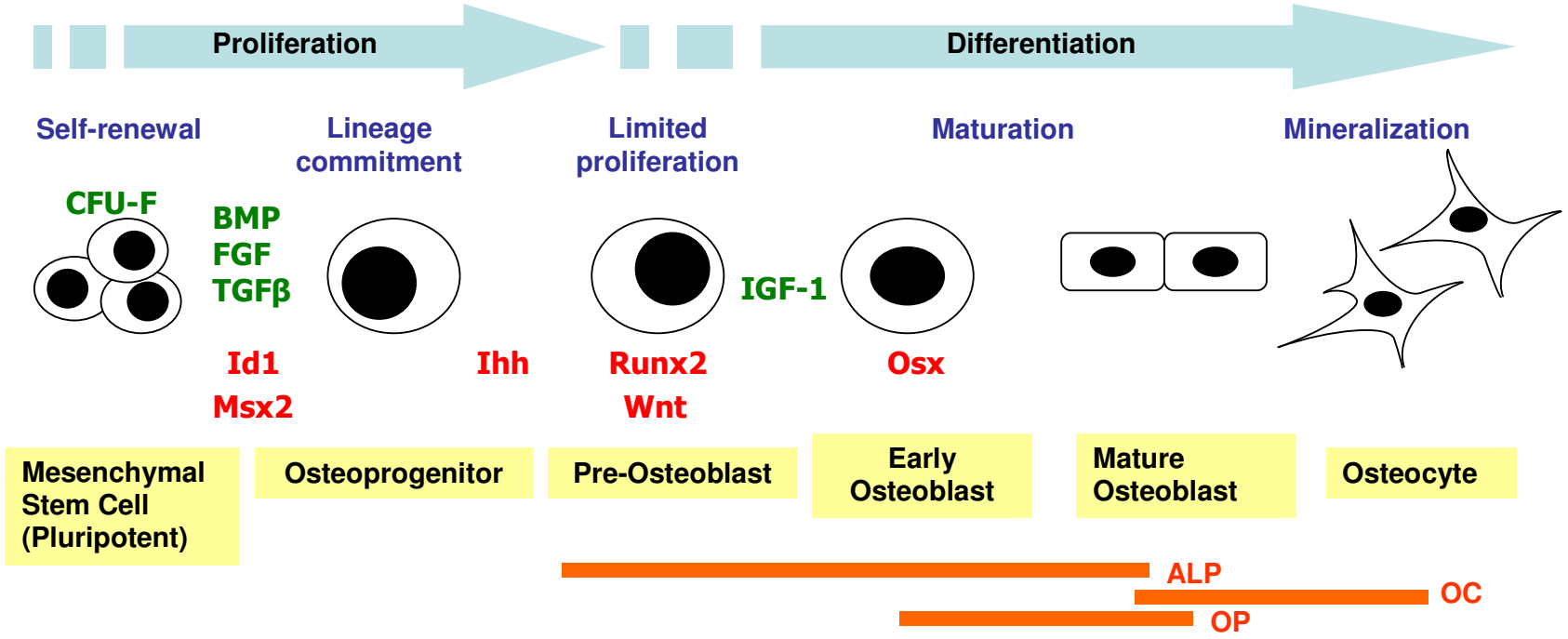


Figure 1.5. The various stages of cell fate commitment to osteoblast differentiation. Growth factors and cytokines (in green) are placed from the start of their expression, transcription factors (in red) and the expressed biochemical markers by osteoblasts (in orange).

importance to osteoblasts as the transcription of key genes Runx2 and osterix are dependent on it. Activation of these 2 master genes drives the terminal differentiation of osteoblasts, by enhancing osteoblast specific genes such as alkaline phosphatase and osteocalcein. In addition, other growth factors may act to complement or antagonize BMP signaling. TGFβ can also induce Runx2 expression via activated effectors Smad2 and Smad3 (Lee *et al*., 2000). Growth factors FGF, IGF-1, and endothelin-1 (ET1) activate osteoblast MAPK (mitogen activated protein kinase) cascade via transmembrane or G-protein coupled receptors. These activated pathways can also ultimately lead to Runx2 and osterix gene regulation, via other kinases such as protein kinase C (PKC) in the case of FGF signaling (Kim *et al*., 2003). Also of importance is the canonical Wnt signaling pathway, whereby co-receptor LRP5 was shown to be involved in bone formation (Boyden *et al*., 2002), independent of Runx2 (Kato *et al*., 2002), while β-catenin was also required for osterix expression (Rodda and McMahon, 2006). Notably, the most well studied connection between osteoblasts and osteoclasts comes from the RANKL-RANK-OPG interaction that show osteoclasts need osteoblast secreted RANKL (receptor activator of nuclear factor kappa-B ligand) to mature (Russell *et al*., 2006).

Of newer interest is osteoblast apoptosis, where the activation of apoptotic pathways may be part of osteoblast signaling since bone cells may undergo programmed cell death to control life span, affecting changes in bone turnover as well (Manolagas, 2000). Proapoptotic proteins Bim, Bak and Bax were found to be induced in osteoblasts via the caspase 3 pathway upon serum deprivation (Liang *et al*., 2008). Nevertheless, the multiple pathways an osteoblast can support due to the abundance of growth factors it secrete can also be a bane as in the case of frequent metastatic

secondary tumors of bone developed from primary adenocarcinomas, through stimulation of osteoblast proliferation (Logothetis and Lin, 2005).

1.3.4 Disorders of bone remodeling

In healthy adults, bone turnover is maintained at a steady state as the rate of resorption roughly equals the rate of bone deposition. Disorders of bone remodeling occur as a result of an imbalance in these two actions. Osteoporosis develops when the rate of bone resorption exceeds the rate of bone formation, such that resorption cavities left by osteoclasts are incompletely replaced by new bone, creating perforations in bone structure. Less common is osteopetrosis, characterized by high bone mass (osteosclerosis) due to the higher bone formation rates, the opposite outcome of the former (Russell *et al*., 2006, Lazner *et al*., 1999)

The cellular mechanism behind bone disorders is not completely understood; as the understanding on bone remodeling remains vague. Bone remodeling is a continuous process with each phase requiring different time frames; resorption takes about 2 weeks in contrast with bone formation needing up to 4 months for complete structural bone replacement (Hadjidakis and Androulakis, 2006).

1.3.4.1 Osteoporosis, a disease of low bone turnover

Osteoporosis/osteopenia is a disease characterized by low bone mass. The most common and widespread bone disease, osteoporosis affects mainly the older population with a higher incidence in women.

One of the two primary causes is loss of gonadal function/estrogen deficiency termed postmenopausal bone loss, which is usually rapid. The other is gradual bone loss with aging (non-hormonal), in which both causes are known to operate through independent mechanisms. Bone loss through aging differs from hormonal deficiency as bone formation decreases over time, due to a progressive decline of osteoblasts supply relative to the demand. This "exhaustion" or senescence of precursor cell population in the marrow results in irreversible loss of bone mass over time, usually affecting cortical bones. Post-menopausal osteoporosis, on the other hand, is effectively the result of excessive osteoclast activity that mainly affects trabecular bones (Manolagas and Jilka, 1995).

Age-related bone loss is also termed as senile osteoporosis. The major sources of senile osteoporosis stem from either systemic abnormality, or osteoblast dysfunction. Systemic influences include insufficient active vitamin D. Intrinsic osteoblast dysfunction results from factors found in the osteoblast or surrounding it (within its microenvironment). The local microenvironment of the osteoblast is controlled by cytokines and growth factors such as BMPs and IGF-1, though none of these factors can solely be responsible for the etiology of aging-associated bone loss (Kawaguchi, 2006).

Therefore, what causes senile osteoporosis? Several genetic and systemic factors have been implicated, in an attempt to uncover its pathophysiology. Osteoporosis is generally known to be a multiple-factor disease and is believed that genetic susceptibility plays substantial role at an early age. Despite no single gene have been identified to be directly the sole cause of osteoporosis, linkage studies have identified

several loci that may contain the genes regulating bone mineral density (BMD), which can alter one's risk to disease progression (Russell *et al*., 2006). One such example of genetic impact is the role of *Sca1* gene, present on a subset of bone marrow stroma whereby *Sca1*$^{-/-}$ mice born with normal bone development matures to senile osteoporosis with age, due to the deficiency in self-renewal capacity of mesenchymal progenitors (Bonyadi *et al*., 2003).

The increased risk for fractures and the lack of promising therapeutics remains the major concerns about osteoporosis (reviewed in Canalis *et al*., 2007). Current drugs are created as pre or post-therapeutics. Anti-resorptive drugs include oestrogens, bisphosphonates and selective estrogen receptor modulators (SERMs). The next class of therapeutics is anabolic drugs, targeted to promote bone formation and these include PTH/teriparatide and strontium (reviewed in Russell *et al*., 2006). More promising osteoblast-promoting drugs may be in the pipeline to enhance bone formation. Local administration of rhIGF-1 could significantly induce new bone formation in the already impaired bone formation ability of aged mice, hence rhIGF-1 may improve senile osteoporosis (Fowlkes *et al*., 2006). Also, small-molecule PYK2 (proline rich tyrosine kinase 2) inhibitor was targeted as a promising anabolic approach to regulate osteoprogenitor differentiation (Buckbinder *et al*., 2007).

1.3.4.2 Mouse models of senile osteoporosis

Senile osteoporosis is difficult to study as it develops slowly, and despite the advances in human and mouse genetics, skeletal physiology remains poorly understood. To date, a database of over 120 bone-related mouse models have been archived to close

the gaps on understanding bone biology. Transgenic and mutant mice remain the most appropriate genetic tools to dissect bone disease (Pogoda *et al*., 2005).

Notably, much of aging-related research came from the SAM (senescence–accelerated mouse) family of mice. In as early as 1970s, Takeda and team, through selective inbreeding of AKR/J strain mice that exhibited natural spontaneous senescence and aging-like features, developed 14 senescence-prone inbred strains and 4 senescence-resistant inbred strains (SAMR) as control counterparts that show normal aging (Takeda, 1999). In particular, the SAMP6 strain developed distinct senile osteoporotic features, and was subsequently used as a model to study the basis behind aging-associated osteoporosis (Matsushita *et al*., 1986). Research on SAMP6/Ta uncovered the depletion of osteoblast progenitor pool as a result of bone marrow tendency to favor adipocyte differentiation, rather than osteoblasts (Suda *et al*., 1994). Also, it was found that the initial healthy numbers of bone marrow osteoprogenitor cells at 1 month of age deteriorated to a palpable 3-fold reduction by the 3rd to 4th month of SAMP6 lifespan (Jilka *et al*., 1996). Hence it is highly probable that the switch in the differentiation program of multipotential mesenchymal progenitors is one such basis for senile osteoporosis, as osteoblasts and adipocytes share the same progenitor. Using the SAMP6 mice, Takeda and team was able to treat senile osteoporosis by a novel surgical method of bone marrow transplant and normalize the defective bone marrow constituents (Takada *et al*., 2006).

More current genetic approaches to understand senile osteoporosis come from mouse models focused on systemic influences of bone metabolism. Circulating levels of IGF-1 could regulate bone growth and density (Yakar *et al*., 2002) and is involved in

the pathophysiology of osteoporosis via IRS (insulin receptor substrates). IGF-1 signaling requires the essential adaptor molecule IRS to activate PI3K/AKT and MAPK pathways (Kadowaki *et al*., 1996). The low bone turnover of *IRS-1*$^{-/-}$ revealed its essential role in promoting catabolic and anabolic osteoblast functions (Ogata *et al*., 2000), while decreased bone formation and increased bone resorption rate of *IRS-2*$^{-/-}$ revealed an uncoupling status that has to be fine-tuned by IRS-2 (Akune *et al*., 2002). In addition, *PPARγ*$^{+/-}$ mice exhibited high bone mass by stimulating osteoblastogenesis from bone marrow progenitors, increasingly prominent with age. Hence, heterozygote mutation of PPARγ, a key regulator of adipocyte differentiation and a factor intrinsic to osteoblasts, shows the fundamental relationship between osteoblasts and adipocytes in the regulation of osteoprogenitor population (Kawaguchi, 2006). Another interesting example of systemic impact on osteopenia comes from the klotho homozygous null (kl/kl) mice that exhibit age-related bone loss and independent impairment of osteoblast and osteoclast differentiation (Kawaguchi *et al*., 1999). These 3 newer players depict the coupling of bone metabolism with bone aging. The role of ECM proteins in osteoporosis was also explored with the study on non-collagenous ECM protein biglycan (Bgn), whereby *Bgn*$^{-/-}$ mice developed osteoporotic-like phenotypes (Xu *et al*., 1998).

1.4 Bone morphogenetic proteins

The bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGFβ) superfamily of signaling molecules which also consist of TGFβ, activin and inhibin (Wozney, 1988). BMPs were first discovered to induce ectopic bone formation when implanted into rodents, and subsequently established an ability to drive undifferentiated stem cells into the osteogenic program (Urist, 1965). The early developmental stages of patterning and organogenesis were found to be regulated by BMPs, and due to its pleiotropic actions it is also involved in adult tissue homeostasis Hogan, 1996).

BMPs proteins have seven cysteine residues (conserved in TGFβ superfamily), and are first synthesized as large precursors that require proteolytic cleaving to release its active C-terminal domain. BMPs can then be secreted as mature, dimeric ligands of 20 – 30 kDa in size. Over 20 distinct BMPs have been isolated. *In vivo* bone promoting BMPs are BMP2, 4, -6 and -7(Osteopontin-1) and -9. Of these, BMP2 and BMP4 remains the foremost well characterized members with potent osteoinductive activities, and has been developed for orthopedic clinical applications in animal models (reviewed in Chen *et al*, 2004). The essential roles of BMP2 and BMP4 in development were validated by the observed embryonic lethality of *bmp2*$^{-/-}$ mice with cardiac defects (E7.5-10.5) and *bmp4*$^{-/-}$ mice with defects in mesoderm formation (E6.5-9.5) (Zhang *et al*., 1996, Winnier *et al*., 1995).

1.4.1 BMP-Smad pathway

The canonical BMP pathway consists of a signaling cascade mediated by serine/threonine (S/T) kinase molecules. The step-wise activation of downstream molecules requires S/T phosphorylation. **Figure 1.6** describes the various signal mediators involved. BMP signaling begins at the plasma membrane, with BMP ligand binding to type I and II BMP receptors, inducing heterodimerization of these receptors. This activates the BMP type II (BMPRII) receptors, which in turn phosphorylates Type I receptors (BMPRI) at its unique GS domain. Upon GS box activation, BMPRIs becomes activated by autophosphorylation and recruits the next step of transducers, the Smads. BMP-Smads (Smads1/5/8) are then activated and form hetero-trimeric complexes with Smad4 (a common Smad shared by all TGFβ superfamily members), to translocate into the nucleus and activate the transcription of target genes (Massague, 2000). **Figure 1.7** illustrates the BMP signaling pathway and the modulators at different compartments of the cell.

In the TGFβ superfamily, there are 5 type II receptors and only BMPRII specifically binds to BMP. 7 type I receptors exist, and BMPRIA (ALK3) and BMPRIB (ALK6) are specific for BMPs, The basic structure for BMP receptors are modeled on TGFβ receptors, seen in **Figure 1.8**. Both type I and type II consist of a short extracellular domain, a transmembrane domain and a large intracellular S/T kinase domain. Type I receptors contain a serine-glycine rich domain (GS box) at the N-terminal region prior to kinase domain, which is needed for activation by BMPRII (Massague *et al.*, 1994, Shi and Massague, 2003). BMPRII has an extremely long C-terminal tail, which was reported to mediate other kinase dependent pathways.

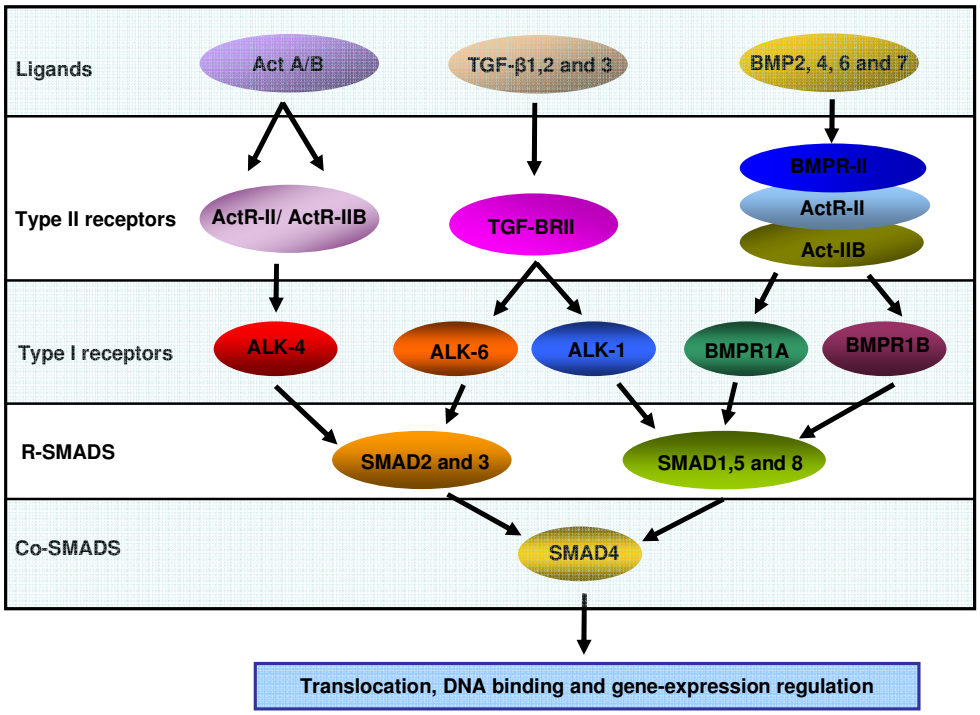


Fig. 1.6. The TGFβ superfamily of signaling molecules. Step-wise activation of phosphorylation casade begins from ligand binding to type II receptors.

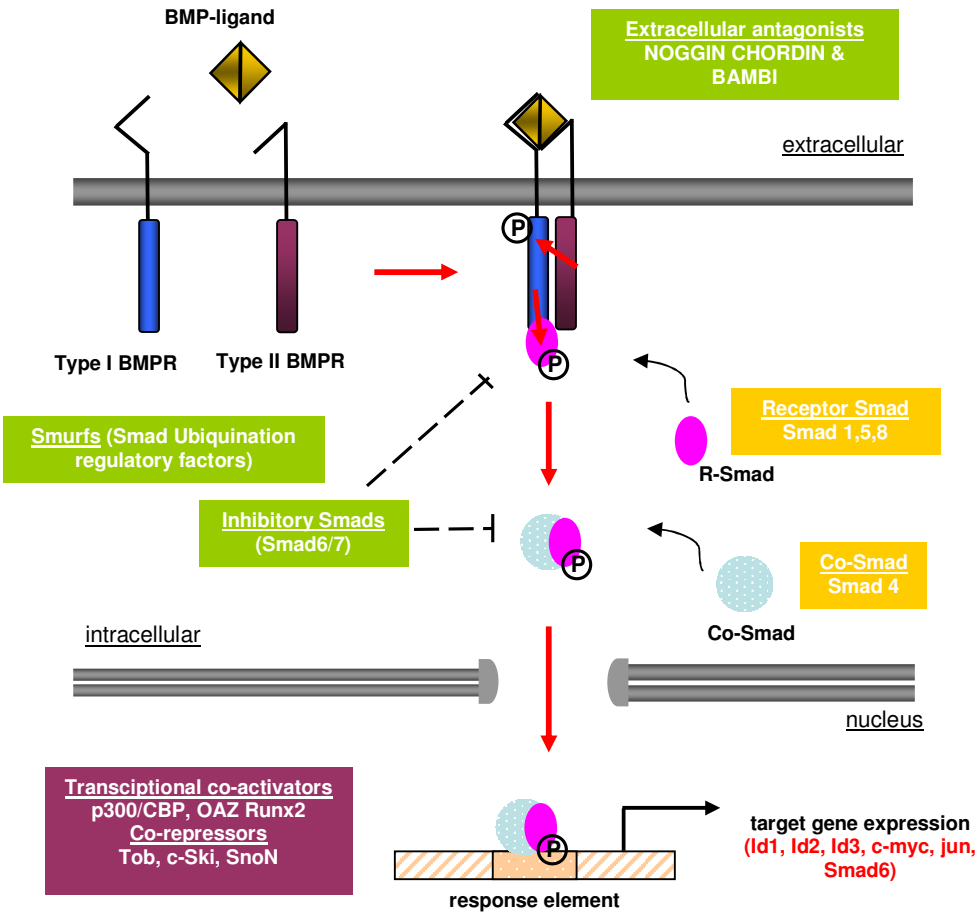


Fig 1.7. The canonical BMP-Smad pathway and signaling modulators. Negative regulators exist at the extracellular and cytoplasmic compartments (green boxes), and as transciptional co-repressors in the nucleus.

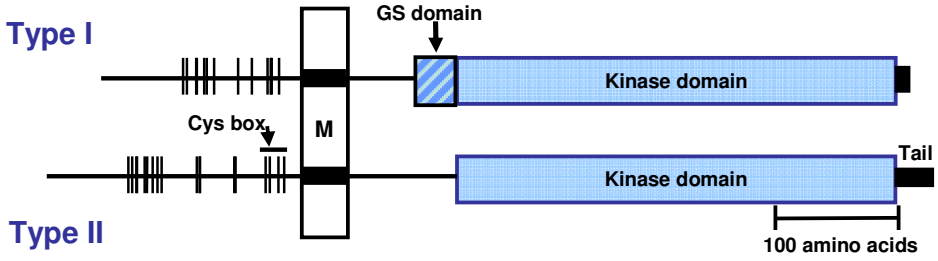


Fig 1.8. The basic structures of BMP type I and type II S/T kinase receptors. Common to these two classes of receptors are the extracellular domain, a single transmembrane domain (denoted M), and a kinase domain.

BMPRIA is a 55 kDa protein that is ubiquitously expressed in most cell types including osteoblasts, and in cultured cells COS7 and osteoblastic cell line MC3T3. This was also the case for BMPRII, a 70-80 kDa protein, which was highly expressed in most tissues, while BMPRIB expression was confined to limited tissues such as glioblastomas. Core BMP signal transduction appears to mediated by BMPR1A and BMPRII rather than BMPRIB, as *bmpr1a*$^{-/-}$ embryos die at E9.5 with no mesoderm formation (Mishina *et al*., 1995), and BMPRII null mice is also embryonic lethal (Beppu *et al*., 2000). On the other hand, mice lacking BMPRIB are viable, albeit with defects in cartilage formation (Yi *et al*., 2000). Even though BMPRIA can bind to BMP ligands in the absence of BMPRII, its binding affinity is facilitated by the presence of BMPRII (Massague, 1996, Kawabata *et al*, 1998).

Smads are the only known direct effectors of the BMP pathway. There are 8 mammalian Smads, and these intracellular BMPR substrates are grouped into three classes, the receptor-Smads (R-Smads, Smads1/5/8), which confers specificity in transcriptional response, the common Smad (co-Smad, or Smad4), which serves as an adaptor for R-Smads, and lastly the inhibitory Smads (Smad6/7), which are discussed in section 1.4.2. R-Smads and Co-Smad contains the conserved MH1 and MH2 domains, bridged by a linker region, while the MH1 domain is missing in I-Smads. The linker region of Smads have been found to be important for functional regulation by other S/T kinases,. More importantly, the C-terminal tail extending out of the MH2 domain contains the site for BMP receptor activation of Smads. The SXS (Ser-X-Ser) motif is only found in R-smads and increases its affinity for Smad4 when activated (reviewed in Massague *et al*, 2000, Miyazono *et al*., 2001).

BMP signaling is complicated by the crosstalk or convergence of BMP signaling pathway with other pathways. Pathways known to crosstalk with BMPs include the JAK-STAT, Wnt, MAPKs, PI3K/AKT and TGFβ pathways (reviewed in Bubnoff and Cho, 2001). Specificity is highly dependent on cell type contexts. In neural induction, the IGF and FGF works in tandem to counter BMP signals, targeting activated Smad1 (Pera *et al*., 2003). Moreover, non-canonical Smad-independent pathways exist, and may operate in synergy or function to oppose BMP-Smads pathway. For example, PKC and MAPKs could be activated by BMPs (Hay *et al.,* 2001, Miyazono *et al*., 2005).

Deletion of genes of individual BMP signaling molecules in mice have often led to embryonic lethality, due to the key roles they play in embryogenesis. However, in humans, several inherited diseases were found to be linked to natural occurring mutations in the BMP pathway. In FOP (Fibrodysplasia ossificans progressiva), a disabling genetic disorder that promotes aggressive bone growth, ectopic BMP4 was found in patients (Gannon *et al*., 1997). Mutations in Smad 4 were linked to familial juvenile polypopsis, and mutations in BMPRII were implicated in a rare autosomal dominant disorder, familial primary pulmonary hypertension (Lane *et al.,* 2000, Deng *et al*., 2000).

1.4.2 Modulators of BMP signaling pathway

Many regulators exist to ensure BMP signaling can be turned off, fine-tuned or is cell type specific. BMP signaling modulators discussed here are categorized according to their cellular localization: extracellular, cytoplasmic and nuclear.

At the extracellular membrane region, BMP antagonists function to block the binding of BMP ligand to its cognitive receptors. BMP antagonists behave in a similar fashion to BMPs as they share the cysteine-knot structure and originate as a secretary signal peptide. The three sub-families of BMP antagonists are based on their cysteine-knot size (Avsian-Kretchmer and Hsueh, 2004) and they are the DAN family (e.g. gremlin, sclerostin and USAG-1), twisted gastrulation (Tg), and chordin/noggin (reviewed in Yanagita, 2005). Of interest are the noggin/chordin family that have been studied in depth, due to their importance in regulating bone formation and turnover. Noggin is a 32 kDa secreted glycoprotein that binds to BMP2 and BMP4 with high affinity and BMP7 with low affinity. Noggin gene was found to be induced by BMP themselves, which reveals the likelihood that noggin could function as a direct and efficient antagonist, and plays a major role in autonomous BMP-signal regulation. The lack of noggin function is deleterious, as noggin homozygous null mice had excess cartilage and present with a failure to initiate joint formation (Brunet *et al*., 1998). In humans, heterozygous mutations cause apical joint fusions, exhibiting noggin haploinsufficiency (Gong *et al*., 1999). Also, transgenic mice with targeted overexpression of noggin in osteoblasts became osteoporotic (Wu *et al*., 2003). Hence, noggin appears to regulate postnatal bone homeostasis as well. Chordin, another antagonist found in the Spemann organizer, is a large protein of 120 kDa containing 4 cysteine rich domains (Piccolo *et al*., 1996). It only binds specifically to BMP2 and BMP4. Mice deficient for chordin display early lethality and a ventralized gastrulation phenotype (Bachiller *et al*., 2003). Furthermore, double knockout mice for noggin/chordin exhibit severe defects in facial structures, head development, partial mesoderm development and abnormal left to right patterning (Bachiller *et al*., 2000). BMP receptors can also be targeted. BAMBI is a transmembrane protein

structurally related to type I receptors, but lacks the serine/theonine kinase domain, thus function as a pseudo-receptor to suppress BMP signaling (Onichtchouk *et al*., 1999). Dullard, a neural gene, could bind and abolish BMP-mediated activation of type I receptors. More importantly, it promoted BMP receptor internalization and ubiquitin-mediated proteosomal degradation via the lipid-raft caveolar pathway (Satow *et al*., 2006).

In the cytoplasm, direct negative mediators of Smad signaling are the third class of Smads, the inhibitory smads I-Smad6 and I-Smad7. Smad6 binds to BMP type I receptors to inhibit activation of Smad1 and Smad5 and consequently prevent heteromerization with Smad4, terminating Smad signaling upstream of receptor Smads. Smad 6 is a BMP target gene and thus participates in BMP signaling feedback. Smad7 is less specific as it can also inhibit TGFβ/activin signaling (reviewed in Miyazono *et al*., 2005). Other regulators of Smad proteins are also required. In osteoblasts, anti-proliferation protein Tob (transducer of ErbB2) associates with Smad1 and Smad5 to inhibit BMP-Smad dependent transcription, while Tob knockout mice displays osteosclerotic phenotype, with increased bone mass in adulthood (Yoshida *et al*., 2000). Smurfs are members of the HECT type E3 ligases that mediate the degradation of receptor Smads (Zhu *et al*., 1999), and enhanced bone formation was observed in Smurf1 null mutant mice. Phosphatases of Smads are the emerging trend towards understanding Smad regulation. The S/T phosphatase PPM1A could dephosphorylate TGFβ-Smads (Lin *et al*., 2006), and was found to deactivate BMP-Smad1 *in vitro* and *in vivo* as well (Duan *et al*., 2006). RNAi-based genetic screening has also identified pyruvate dehydrogenase phosphatases (PDP) as BMP specific S/T phosphatases (Chen *et al*., 2006).

In the nucleus, the specificity of BMP response comes into play, with the myriad classes of transcription factors/DNA binding proteins that function as co-activators, or co-repressors to complex with Smads on BMP target genes. Much of the transcriptional regulators is also shared by the TGFβ family, and perhaps differs in response by cell type expression. Transcription factor Runx2, is essential to induce genes involved in bone formation. Ski and SnoN interacts with Smads, and are transcriptional co-repressors that induce histone deacetylation (Wan and Cao, 2005). Co-activator p300/CBP was found to bridge STAT3 and Smad1 and transduce BMP-LIF-mediated transcription in fetal brain (Nakashima *et al*., 1999). In addition, small C-terminal domain phosphatases were found to act as nuclear Smad phosphatases (Knockaert *et al*., 2006). Nucleoplasmic shuttling adds another level of complexity in the modulation of Smad signaling (reviewed by Xu and Massague, 2004). Smad6 was found to have a novel function in the nucleus through interaction with co-repressor CtBP to repress BMP-induced Id1 transcription (Lin *et al*., 2003).

1.4.3 BMP target genes

Genes targeted by BMPs regulate transcriptional responses to bone differentiation, skeletal patterning and angiogenesis. Conversely, BMPs potently repress genes involved in muscle differentiation (such as MyoD and myogenin), since they direct the differentiation route of mesenchymal stem cells away from the osteogenic program. Genes that are direct targets of BMPs include Id proteins, Dlx5, Tsg, Msx1/2, GATA2, Tbx2, collagen, Smad6 and Smad7. Mostly BMP modulators themselves, they are rapidly transcribed due to the SBE (Smad binding elements) sites found on their promoters. On the other hand, indirect target genes such as osterix and osteocalcin require *de novo* protein synthesis, but are indispensable for BMP mediated

functions (Miyazono *et al*., 2005). The Id proteins remain the most well-characterized target genes and are further discussed below.

1.4.3.1 Id proteins

Id (inhibitor of differentiation, or inhibitor of DNA binding) proteins are a subclass of the basic helix-loop-helix (bHLH) family of transcriptional regulators that are critical in development. All HLH proteins contain the E-box consensus motif CANNTG that regulate distinct tissue specific gene transcription. Dimerization of HLH proteins is required for activation, as the formation of four helix bundles allow its basic region to bind DNA (Massari and Murre, 2000). Id proteins are a special subclass of the HLH family as they possess the HLH domain but lack the basic DNA binding domain. As a result they function as dominant negative regulators by binding to bHLH members and abolish dimerization, thus antagonizing transcription as illustrated in **Figure 1.9A**. Therefore, BMP-induced Id proteins promote cell proliferation and block differentiation programs of many cell types (Norton *et al*., 1998). Ids are proposed to act as molecular "brakes" needed for proper and timed developmental processes (Carmeliet, 1999), and when this is breeched, excess Id1 production leads to uncontrolled proliferation and possibly cancer, while the loss of Id1 increases the likelihood of senescence with $p16^{INK4a}$ up-regulation (reviewed in Yokota and Mori, 2002, Perk *et al*., 2005). Id proteins targets not only bHLH transcription factors such as E-proteins, but also Ets family members, and Rb. Hence its roles are not limited to development, but also in balancing cell proliferation versus survival and senescence versus cancer (reviewed by Zebedee and Hara, 2001, Sikder *et al*., 2003). **Figure 1.9B** illustrates Id1 role in senescence.

Four mammalian isoforms Id1 to Id4 have been identified, and while they exhibit some functional redundancy, each Id protein have been shown to retain unique roles, which explains their distinct expression patterns throughout embryogenesis (Jen *et al.*, 1997). Gene ablation studies on Id proteins reveal the *in vivo* overlapping functions of Id1 and Id3, as each $Id1^{-/-}$ and $Id3^{-/-}$ mice are normal, yet double knockout $Id1^{-/-}Id3^{-/-}$ is embryonic lethal. Any other Id combinations of double knockout mice were also reported to be non-viable (Ruzinova and Benezra, 2003). Moreover, the essential role for Id1 and Id3 in *in vivo* bone formation was studied using Id1/Id3 heterozygous knockout mice (Maeda *et al.*, 2004).

In molecular studies, Id1 is often the candidate BMP target gene analyzed. The Id1 gene can be rapidly transcribed and translated within 30 mins of BMP induction. This efficient responsiveness to BMP is due to distinct SBE motifs found on Id1 promoter (Korchynskyi and Ten Dinjke, 2002), facilitated by direct binding of Smad1 and Smad4 to promoter as well (Lopez-Rovira *et al.*, 2002). The serum responsiveness of Id1 to growth signals are found to be regulated by a complex containing the immediate-early response (Egr-1) gene (Tournay and Benezra, 1996).

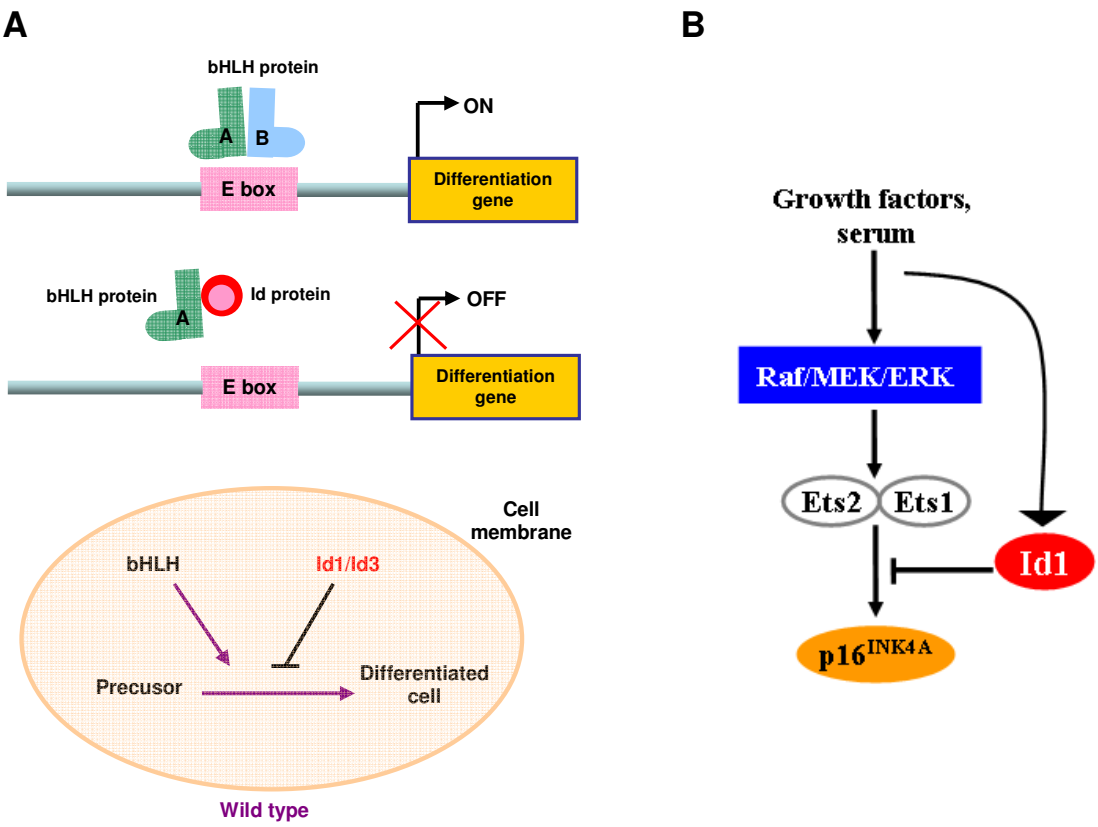


Fig 1.9. Id1 in diffentiation and cell growth. **(A)** Mechanism of Id1 in inhibiting differentiation. **(B)** Id1 opposes the effects of Ets, which represses p16 and induced p16-mediated senescence.

1.5 Thesis rationale

Since the discovery of c-Abl, there has not been much progress in understanding its function, the genes it affects, and the roles it plays in cell signaling networks. The establishment of c-Abl knockout mouse provided genetic evidence for its role in development and survival. Also, many of the *c-Abl*$^{-/-}$ phenotypes observed resembled aging-related syndromes.

This study was undertaken to investigate the molecular mechanisms behind the premature senescence phenotype of *c-Abl*$^{-/-}$ osteoblasts. c-Abl mice showed reduced bone formation rate, thinner cortical bone and lower trabecular bone volume. It was found that defects in osteoblast maturation were the underlying causes of its osteoporotic feature (Li *et al*., 2000).

My project employs mainly primary osteoblasts, as well as bone sections to help identify the pathways and key mediators involved. In doing so, the *ex vivo* and *in vivo* results provides a better insight into the understanding of physiological functions of c-Abl in bone development, bone homeostasis, and how it participates in a signaling pathway. The consequences of altered tyrosine kinase activity in the case of BCR-ABL, and its impact on the BMP signaling pathway are also explored here.

This thesis is divided into 9 chapters;

Chapter 1 contains general literature review to provide background information for this project.

Chapter 2 describes the materials and methods used for this study.

Chapter 3 describes the poor growth potential and premature senescence of *c-Abl*$^{-/-}$ osteoblasts through the up-regulation of p16^{INK4a}.

Chapter 4 identifies the two major regulators of p16^{INK4a}; Id1 and MAPK-ERKs, and their expression pattern in *c-Abl*$^{-/-}$ osteolasts.

Chapter 5 examines in detail c-Abl regulation of Id1 expression under the BMP pathway, and the positive effect of c-Abl on Smad1/5/8 activation.

Chapter 6 demonstrates how c-Abl regulates BMP signaling through the phosphorylation of BMPR1A, and the mechanism behind compromised Smad activation in the absence of c-Abl.

Chapter 7 deals with the negative effect of c-Abl on BMP-induced ERK activation, through its action on BMPR1A.

Chapter 8 provides another perspective on c-Abl role in osteoprogenitor expansion, using the BCR-ABL model to study BMP-Id1- p16^{INK4a} signaling and the implications for CML. The expression of p16^{INK4a} in bone marrow cells relative to age was also explored.

Chapter 9 is for general discussion and future perspectives.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and antibodies

Table 2.1 List of antibodies used

Antibody	Clone / Host	Brand/company
Id1 $p16^{INK4a}$ TAK1	Polyclonal/rabbit Polyclonal/rabbit Monoclonal/mouse	Santa Cruz
p-Smad1/5/8 p42/p44 MAPK p-p42/p44 MAPK TAB1 p-TAK1 (Thr184/187) p-MEK1/2 MEK1/2	Polyclonal/rabbit	Cell Signaling
HA affinity matrix	Monoclonal/ rat	Roche
Flag-M2 affinity gel Actin	Monoclonal/ mouse	Sigma
BMPRII BMPRIA	Polyclonal/ goat	R & D Systems
BMPRIA BMPRIB	Polyclonal/rabbit	Zymed/Invitrogen
c-Abl (Ab-3) Smad1	Monoclonal/mouse Polyclonal/ rabbit	Oncogene/Calbiochem
p-Tyrosine (PY20)	Monoclonal/mouse	BD Transduction Laboratories

For drugs and chemicals used, rhBMP2 was obtained from iDNA, rmNoggin and rmChordin from R&D Systems, hygromycin-B from Invivogen, puromycin dihydrochloride from USBiological, sequabreene from Sigma, U0126S from Promega and Imatinib mesylate/STi571 from Norvartis, tunnel assay kit and BrdU labeling reagent from Roche. Formulations and chemical/drug preparations can be found in Appendix E.

2.2. Cell culture

Adherent cell lines C2C12, cos7, *c-Abl*$^{-/-}$ and *c-Abl*$^{-/-}$ *Arg*$^{-/-}$ mouse embryonic fibroblasts were cultured using Dulbecco's Modified Eagle's Medium (DMEM, Sigma). Suspension cells HL60 and K562 were cultured using RPMI-1640 media (Sigma). MC3T3E1 and *c-Abl*$^{-/-}$ or wild type primary osteoblasts were cultured in Minimal Essential Medium Alpha (αMEM, Gibco), with 15% FBS for osteoblasts. All media were supplemented (otherwise stated) with 4.5g/L glucose, 2mM L-glutamine (Gibco), 10% (v/v) fetal bovine serum (FBS, Hyclone), anti-mycotic antibiotic solution containing 100 units/ml penicillin, 100ug/ml streptomycin and 250ng/ml amphotericin-B, and 20mM HEPES in a humidified 37^{o}C incubator containing 5% CO_2.

2.3. Cavarial osteoblasts isolation and culture

c-Abl null mice (abl^{1}) were crossed to C57BL/6 mice six times before use. To extract primary osteoblasts, calvaria of 19-20 day old fetuses were harvested, washed in PBS before being digested for 10mins with collagenase and dispase (Roche) in αMEM at 37^{o}C. Digestion was repeated for another 3 times. The supernatant from the first digestion was discarded and last 3 digested supernatants pooled. Finally, the cells were then washed and plated onto 6-well plates and left to grow in αMEM supplemented with 15% FCS until they reach confluency. The pre-osteoblast cultures were then trysinized and cell population expanded to passage 3 before being used for experiments. c-Abl deficient mice were compared to their control littermates in all experiments.

2.4. Senescence associated-beta-galactosidase (SA-β-Gal) assay

Cells were washed in TBS buffer, and then fixed in 2% formaldehyde / 0.2% glutaradehyde for 3 mins at room temperature. Cells are washed with PBS again, and rinsed with H_2O to remove fixation solution. Freshly prepared X-Gal staining solution (Appendix C) was then used to stain cells for 2 hrs at 37^{o}C. β-Gal positive cells were counted.

2.5. Cell proliferation assays

The following 2 methods were employed;

Manual cell counting

The proliferation potential of the mutant and wild type primary osteoblasts starting from passage 3 were used for continuous counting and plating via the trypan-blue dye exclusion method. $4x10^5$ cells were plated onto 6 cm dishes, cultured for three days, and counted. The same number of cells were re-plated, cultured for three days and counted again. The procedure was repeated until the cells stopped dividing.

BrdU incorporation

Cell proliferation was directly assessed using DNA synthesis as an indicator of growth by measuring the incorporation rates of synthetic DNA analogue BrdU (5'-Bromo-2'-deoxy-uridine) in place of thymidine. The Cell Proliferation Biotrak ELISA kit (Amersham Biosciences) was used and apart from following manufacturer's protocol; osteoblasts at various passages were seeded at 3 different densities of $1x10^3$, $5x10^3$ and $1x10^4$ in 96-well plates, in triplicates. The next day, BrdU labeling was carried out and incubated overnight (16 hrs) and the final colorimetric measurement at A_{450nm} (reference A_{690}nm) using a plate reader.

2.6. Infection by retrovirus and selection

Transfection

Platinum-E cells (PlatE, courtesy of Dr. Kitamura), a modified 293T-derived ecotropic packaging cell line, was seeded in a 10 cm^2 dish to achieve 80% confluency the next morning (refer to Appendix B for PlatE media). 6ug of retroviral plasmid harbouring Id1, GFP, $p16^{INK4a}$, c-Abl or BMPR1A and control empty vector was tranfected into PlatE cells overnight using Fugene reagent (Roche). The following day, cells were replaced with 8mls of fresh media, and left to incubate for another 48-72 hrs.

Infection

Following incubation, the media supernatant containing matured virus was used to infect early passage primary cells. Polybreene (Sequabrene, Sigma) was added (final concentration 8ug/ml) to viral supernatant and filter-sterilized prior to infection. Post 8 hrs infection, fresh media containing polybreene was added to dilute existing supernatant at a 1:1 ratio and left for a further 16 hrs. Supernatant was then aspirated and cells recovered in fresh media for 24 hrs before starting the selection stage.

Selection

24 hrs post recovery, cells were subjected to selection with hygromycin B (HygroGold, InvivoGen) or puromycin (US Biological).The selection process was monitered daily to observe for cell death, so as to optimize drug dose till a ~30-40% cell death rate was achieved, and after 3-7 days in selection (with selection drug changed every 3 days), resistant cells were further passaged and seeded for various experiments. Levels of protein overexpression were observed by western blotting.

2.7. Bone marrow extraction

Mice were sacrificed using Advertin as anesthesia and hindlegs removed to obtain femoral (thigh) and tibia bones. The bones are washed several times with cold PBS and trimmed at the edges to reveal the bone marrow. Marrow/stroma was then flushed out using DMEM normal media and a syringe. Marrow cells were then spun down in a 4^{o}C centrifuge and cell pellet washed several times with cold PBS. Cells were lysed with TNEN lysis buffer (for protein), or TRIzol (for RNA extraction). Red blood cells were lysed using RBC lysis buffer prior to beginning protein or RNA work.

2.8. Transient transfection

Cells were counted and seeded to achieve 40-50% confluency the next day. Transfection was carried out with 3-4ug total DNA per 6 cm^2 dish using Lipofectamine and Plus reagents (Invitrogen) for cos7 cells and Fugene (Roche) at a 6:1 ratio for MC3T3 cells, according to manufacturer's protocols. Cells were incubated with transfection complex for an average of 3.5 – 5 hrs (cos7) or overnight for MC3T3, before being recovered with fresh complete media for a minimum of 36 hrs, including serum starvation for the last 16 hrs if necessary. Upon treatment, cells were harvested to proceed with western blotting or RNA extraction.

2.9. Western blot

Sample preparation

Harvested cells were lysed on ice and washed twice with cold PBS. TNEN lysis buffer (containing 50mM Tris, pH7.5, 100mM KCl, 1mM EDTA, 0.5% NP-40, 1mM PMSF, 1mM sodium orthovanadate, 10mM NAF, 1mM β-glycerolphosphate and 10ug/ml each of aprotonin and leupeptin) was then added and lysates left to rock for

30 mins at 4^{o}C before spinning down at maximum speed for 10 mins. An aliquot of spun supernatant clear of debris was used for protein quantitation using the Protein Assay Reagent (Bradford assay) from Biorad. Lysates were then normalized in a fresh eppendorf tube and 5X SDS loading buffer was added before boiling samples for 5 mins at 95 ^{o}C.

SDS-PAGE and detection

SDS-PAGE gels were prepared using the Biorad protean minigel apparatus. Components of these gels can be found in Appendix D. 13.5% -15% gels were used for detecting proteins such as Id1 and $p16^{INK4a}$. 8%-12% gels were used for BMPR1A, phospho-Smad1/5/8, TAB1, TAK1, and 7.5% gel for c-Abl. Gels were run at 80-120V along with protein standards (kaleidoscope marker, Biorad). Transfer by electric current was carried out using PVDF (Millipore) membranes for 2 hrs at 300mA, or overnight at 100mA in 4^{o}C. Thereafter, membranes were stained with Ponceau S to check for integrity of transfer and stain removed with rinsing with TBST buffer (Appendix A). Membranes were then blocked with 5% (w/v) nonfat milk or 5% (w/v) BSA (for goat host, or all bmpr1A, 1B antibodies) for 1 hr before incubation with primary antibody overnight. The next day, primary antibody was removed, membranes washed 3X (5 mins per wash) with TBST, before adding secondary-HRP linked antibody for 1 hr. Membranes were then washed 4X and subjected to ECL solution for detection of protein bands. Varying film exposures were obtained before analysis of results. Membranes could then be stripped and reprobed if necessary. 0.2M glycine solution was used for stripping PVDF membranes, for 15-10 mins and rinsed thoroughly with ddH20 for reprobing.

2.10. Immunoprecipitation

Cells were harvested on ice by washing twice with cold PBS, lysed using CO-IP buffer (Appendix A). Lysates were then left to rock at 4^{o}C for 30 mins. The tubes were then centrifuged at max speed 16100g (or 14300rpm) for 10 mins (Note: all steps and centrifugation was performed at 4^{o}C, with tubes kept on ice). A small aliquot (1-2ul) of supernatant was taken to perform protein quantitation using the Bradford assay with a spectrometer. All OD readings were ensured that they fall within the linear range of 0.1-0.5. Thereafter, OD measurements were calculated and lysates normalized with lysis buffer in a new tube. 25ul of anti-HA affinity beads (Roche) was added to each tube and left to rock for 2-3 hrs. After immunoprecipitation, the beads were spun down at 6000rpm for 8 mins and supernatant aspirated. Beads were subsequently washed 5 times, the first 3 times using CO-IP buffer, and the last 2 washes using cold PBS. Finally, after washing and final aspiration the beads were incubated with 2X SDS-PAGE loading buffer, boiled for 5 mins at 95 ^{o}C and ready to proceed with western blotting.

2.11. RNA extraction

Extraction and phase separation

Prior to extraction, monolayer cells grown on culture dish were washed twice with cold PBS to remove residual media. Likewise, suspension cells (e.g. K562 and marrow cells) were centrifuged and cell pellet washed twice with PBS. TRIzol reagent (Invitrogen) was added (at 1ml per 10 cm^2 of cells), to scrape and the lysate carefully dispersed by going through a 1ml sterile pipette tip 6 times before being placed in an eppendorf tube and left to sit at room temperature (rt) for 5-10 mins to permit complete dissociation of nucleoprotein complexes. Choloform ($CHCl_3$) (at

0.5ml for every 1ml of TRIzol) was then added and tube vigorously hand-shaked for 15 sec and left to sit at rt for another 2-3 mins. Thereafter the sample was centrifuged at 4^{o}C, 12,000g for 15 mins. RNA will be exclusively contained within the colorless upper aqueous phase.

Precipitation phase

The aqueous phase was carefully transferred to a new tube. Precipitation starts with the addition of isopropanol (at 0.7ml isopropanol for every 1ml TRIzol used), mixed by hand and incubated at 10 mins rt before centrifugation at 4^{o}C, 12,000g for 15 mins. A gel-like pellet was then formed at the bottom corner of tube.

RNA recovery

The supernatant was carefully removed, and pellet washed once with 1ml of 75% ethanol, and centrifuged at 12,000g for 10 mins. The RNA pellet was then air-dried at rt till its whitish pellet color turns translucent (approx 15 mins onwards). Pellet was then dissolved using RNase-free water, pipetted gently, and placed in 55^{o}C waterbath for 20 mins. RNA was then measured at A_{260} for concentration, and at $A_{260/280}$ ratio for purity check.

2.12. RNA extraction of femur/long bones

c-Abl adult mice were sacrificed and femur bones carefully removed from skin and muscle tissue using sterile dissection apparatus. The bones were rinsed with sterile cold PBS several times and placed in eppendorf tube, then immediately frozen using liquid nitrogen. Tubes containing long bones were then stored at -80^{o}C. Prior to RNA extraction, the femurs stored at -80^{o}C have to be broken down to powder form. A makeshift table was prepared, using dry ice and pre-chilled autoclaved mortar and

pestle. Femurs were grinded to powder with liquid nitrogen and further kept in dry ice until ready for RNA extraction using Trizol method as in 2.11.

2.13. Reverse Transcription – Polymerase chain reaction (RT-PCR)

Reverse transcription

To assess the mRNA levels of Id1, Semi-quantitative RT-PCR assays were carried out. Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen), quantitated, and then used as template for reverse transcription. Reverse transcriptase AMV, RNase inhibitor, dNTPs and oligodT primers (Roche) were added and incubated at 42^{o}C for 1 hr and thereafter heat inactivated at 90 ^{o}C for 5 mins.

PCR

The synthesized cDNA serves as a template to probe Id1 transcript, using DNA Taq polymerase (Roche). Beta-actin was used as an internal control. The various primers used, annealing temperature and cycle number for PCR are as listed below.

Table 2.2 RT-PCR primers list

Gene	Primer sequence 5' – 3' (F-forward, R-reverse)	Annealing Temperature (^{o}C)	Cycle Number
Id1	F- ACTCACGCCTCAAGGAGCTGG R- TCAGTGCGCCGCCTCAGCGACAC	57	30
p16	F- AGTCCGCTGCAGACAGACTG R- CGGGAGAAGGTAGTGGGTC	55	35
Id3	F- GGTGCGCGGCTGCTACGAG R- CAGGCCACCCAAGTTCAGTCC	56	27
JunB	F– CCACCATCAGCTACCTCCCAC R– TCAGAAGGCGTGTCCCTTGAC	56	30
Smad6	F- GAGAGTGACTGCGAGACGGTGACCTG R- GCGGCCGAGCAGCAGCTGCGG	58	25
Smad7	F- TCCTGCTGTGCAAAGTGTTC R- AGTAAGGAGGAGGGGGAGAC	58	25
BMPRIA	F- TCGTCGTTGTATTACAGGAC R- TTACATCCTGGGATTCAACC	56	35
BMPRIB	F- GCTTTGGACTCATCCTCTGG R- CACTGGGCAGTAGGCTAACG	56	35
BMPRII	F- GGTAGATAGGAGGGAACGGC R- CACTGCCATTGTTGTTGACC	56	35
Actin	F- AGATGTGGATCAGCAAGCAG R- GCGCAAGTTAGGTTTTGTCA	56	25

2.14. Luciferase reporter assay

C2C12 cells were counted and seeded ($0.5x10^5$ per 12-well) to achieve 45% confluency the next morning. Cells were transfected with DNA (total of 1.2ug DNA in equal proportions, along with 5ng TK-null plasmid as internal control) using Lipofectamine and Plus reagents (Invitrogen) for 3.5 hrs, and recovered with fresh complete media. Cells were harvested at an average of 48-72 hrs post-transfection (including starvation and/or BMP2 addition if needed). Harvesting starts from washing cells twice with PBS, adding 150ul 1X Passive Lysis Buffer (Promega) to rock for 20 mins, lysate collected, and spun down at 2000 rpm for 2 mins before reading. 20ul of lysate was used to determine luciferase and renilla activity using the luciferase and STOP and Glo reagents (Promega) respectively, in the TD20e luminometer under the dual-luciferase mode. Ratio of luciferase/renilla activity was obtained and the normalized values plotted on a chart for analysis.

2.15. RNA Interference

siRNA preparation and transfection

Murine MAP3K-TAK1 siRNA (ON-Targetplus SMARTpool L-040718-00-0010) and non-target pool siRNA as control were resuspended in 1X siRNA buffer to a stock concentration of 20nM and stored in aliquots at -20^oC (Dharmacon). C2C12 cells were seeded the night before at a density of $1.2\text{-}1.5x10^5$ per 6-well in antibiotic-free complete media to achieve 70% confluence the next day. siRNA and non-target control were then diluted to 2uM final concentration and transfected using Dharmafect 3 reagent according to manufacturer's instructions and left to incubate for 48 and 60 hrs. Cells were then treated with BMP2 for various time points and harvested for western blot analysis.

The siRNA product - Mouse MAP3K7, NM_172688 (10nmol) consist of 4 pooled duplex primer pairs and target sequences are available online at http://www.dharmacon.com/predesignedsirna/search.aspx.

2.16. Cloning

BMPRIA deletion mutants were PCR-cloned with reverse primers flanking the 30, 35, 45 and 50 kDa regions of full length wild type BMPR1A expression construct (gift from Dr Chen Ye-Guang). PCR products were then gel-purified and digested with XbaI and EcoRI, and ligated into pCDNA-NE vector (N'-terminal HA-tag) using Rapid Ligation Kit (Roche). Transformation into competent *E. coli* (DH5α) was then carried out at 42^{o}C, left to recover at 37 ^{o}C in 0.5ml LB broth (without selection drug) for 1 hr and plated onto ampicillin LB agar plates overnight at 37^{o}C. Single ampicillin-resistant colonies were isolated next day and grown with 4ml LB broth (with ampicillin) overnight. Purification of plasmid was performed with Miniprep Kit (Qiagen) and positive clones confirmed with enzyme digestion (for size) and sequenced using dideoxy termination method.

2.17. Site directed mutagenesis

Using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene), point mutations were created on BMPRIA. PCR was carried out at annealing temperature of 60^{o}C for 18 cycles, followed by extension at 68^{o}C for 12 mins. DpnI was used to remove orginal BMPRIA template DNA (methylated, hence recognized and digested). BMPRIA single tyrosine mutants A to E were cloned from full length BMPRIA as template, while combination mutants F to I were cloned from A-E as templates, at HindIII and NheI sites into pCDNA-NE vector. Mutant plasmids were

then transformed to isolate positive clones and mutations confirmed by sequencing along with BLAST (NCBI) search online.

2.18. Densitometric analysis

To measure the band intensity of blots and set it against control protein/RNA bands for normalization, the Bio-Rad GS-700 imaging densitometer was used. Using the Quantity one program, the bands on X-ray films were boxed and their total intensity volume quantitated against film background. Corrected integrated volumes were taken, and ratio calculated against respective control bands. The arbitrary units were then used for graphical representation.

2.19. Statistical analysis and data presentation

All experiments were performed at least 3 times. Statistical analysis was performed using the student's unpaired *t*-test (STATISTICA).

CHAPTER 3

PREMATURE SENESCENCE OF *C-ABL*$^{-/-}$ OSTEOBLASTS AND THE UP-REGULATION OF p16^{INK4a}, A BIOMARKER OF AGING

3.1 *c-Abl*$^{-/-}$ osteoblasts have decreased proliferation ability and undergoes premature senescence

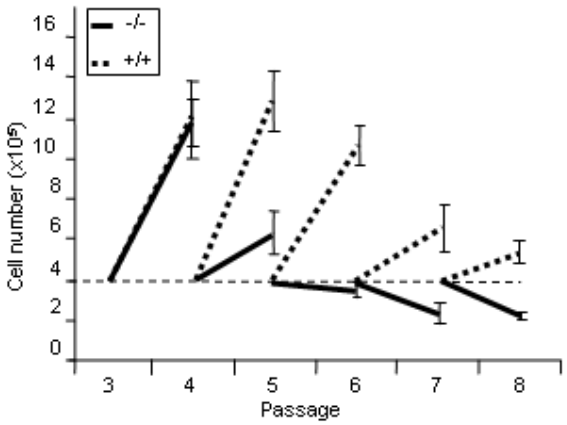
c-Abl$^{-/-}$ mice show features of senile osteoporosis, this phenotype primarily caused by a decline in the function of osteoblasts (Li *et al*., 2000). In addition to the differentiation defects, it was found that *c-Abl*$^{-/-}$ osteoblast cultures were particularly difficult to maintain with increasing passages, and generally senesce after the 4th to 5th passages. This was in contrast to the wild type osteoblasts, which could be maintained past another 3 to 4 more passages. To study the growth potential of these osteoblasts, a modified 3T3 procedure was used (Randle *et al.*, 2001). Osteoblasts were derived from single cavarial bone of an 18-19 day fetus and plated onto a 6-well or 35 mm dish (counted as passage 1). Upon confluence, these cells were further expanded to a 60 mm dish (passage 2) and then to a 100 mm dish (passage 3) before being used for experiments. Cells were counted at every passage (post 3 days) and further re-plated at 4 x 10^5 cells/60 mm dish till the cells stop dividing. We detect little difference in growth rates of c-Abl mutant and wild type osteoblasts of early passages **(Fig. 3.1A)**. However, after the 3rd passage the growth rates differed tremendously, since c-Abl mutant osteoblasts ceased growing at passage 5, while wild type osteoblasts could be maintained till the 8th passage before senescing eventually. The percentage of dead cells was factored in all cell counts and was found to be in similar proportions,

therefore indicating that *c-Abl*$^{-/-}$ osteoblasts contained an innate defect in proliferating capacity.

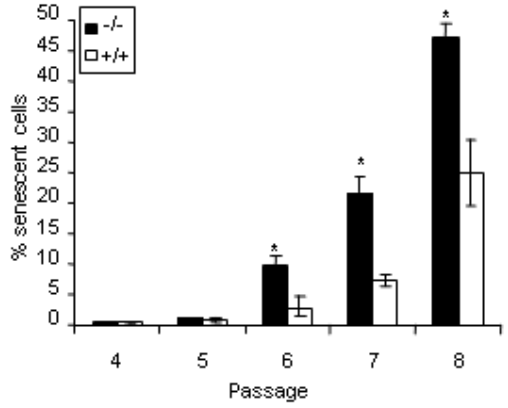
Moreover, staining of senescence associated-β-Galactosidase (SA-β-Gal), a histochemical marker active only in senescent cells (Dimri *et al*., 1995), revealed an increase in percentage of SA-β-Gal positive cells in *c-Abl*$^{-/-}$ osteoblasts with continuous passage **(Fig. 3.1B)**. As alterations in cell metabolism and morphology follow replicative senescence (Ben-Porath and Weinberg, 2004), the increase in SA-β-Gal staining with both mutant and wild type osteoblasts over time reveals that primary osteoblasts undergo senescence, albeit with the mutant osteoblasts at an accelerated rate.

This premature senescence phenotype of *c-Abl*$^{-/-}$ osteoblasts was further supported by the findings that *c-Abl*$^{-/-}$ mice possessed lower osteoblast numbers in their bones **(Fig. 3.1C**). *In vivo* BrdU labeling on E19 embryonic bones validated the lower growth rates as *c-Abl*$^{-/-}$ bones had fewer BrdU positive cells in the S phase of cell cycle, as compared to its wild type counterpart **(Fig. 3.1D)**. Hence, osteoblasts lacking c-Abl have limited lifespan and suffer from accelerated senescence.

A


-/-
+/+
Cell number (x10⁵)
16
14
12
10
8
6
4
2
0
3
4
5
6
7
8
Passage

B


-/-
+/+
% senescent cells
50
45
40
35
30
25
20
15
10
5
0
*
*
*
4
5
6
7
8
Passage

C

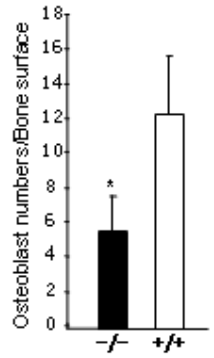

Osteoblast numbers/Bone surface
18
16
14
12
10
8
6
4
2
0
*
−/−
+/+

Fig. 3.1. *c-Abl*$^{-/-}$ osteoblasts do not grow well and senesces prematurely.

(A) Growth of *c-Abl*$^{-/-}$ and wild type osteoblasts. Calvarial osteoblasts were cultured as described and cell count numbers plotted against passage numbers. **(B)** Histochemical staining of SA-β-Gal. Mutant and control osteoblasts were cultured as in (A), then fixed and stained at a neutral pH for β-Gal. Percentage of SA-β-Gal positive cells at each passage was shown. **(C)** *c-Abl*$^{-/-}$ bone sections showed reduced number of osteoblasts. *, $p<0.05$. Femur bones of 2-month old *c-Abl*$^{-/-}$ and wild type mice were used for histomorphometric analysis.

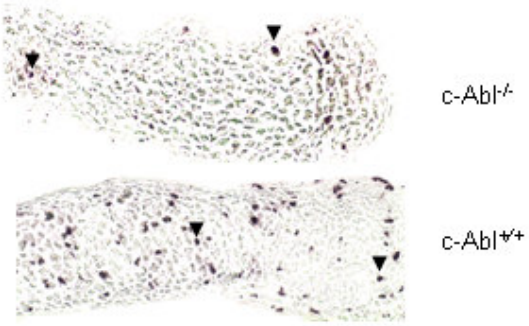


Fig. 3.1. *c-Abl*$^{-/-}$ osteoblasts do not grow well and senesces prematurely. (continued)

(D) Reduced osteoblast proliferation in *c-Abl*$^{-/-}$ mice. BrdU was injected into pregnant mice at E19 for 2-3hours and the mice sacrificed. The embryos were fixed and cryo-embedded and stained for BrdU positive cells in the long bones. (Black arrows indicate examples of positive BrdU labeled cells).

3.2 $p16^{INK4a}$ is up-regulated in *c-Abl*$^{-/-}$ osteoblasts

The two signaling pathways known to mediate senescence are the $p16^{INK4a}$/pRB and the $p19^{ARF}$/p53 pathways (Lundberg *et al*., 2000). In order to distinguish the pathway that governs c-Abl mediated senescence, the expression of key proteins of these pathways were monitored. Osteoblasts were harvested at passage 4 and 6, when mutant cells ceased to divide. Passage 4 cavarial osteoblasts correlate with the time frame whereby considerable difference in total viable cell numbers could be seen between *c-Abl*$^{-/-}$ and wild type osteoblasts **(Fig. 3.1A)**. It was observed that the protein expression of $p19^{ARF}$ and p21 remain unchanged at either passage points in both *c-Abl*$^{-/-}$ and normal osteoblasts, suggesting the lack of significant $p19^{ARF}$ or p21 involvement in mediating *c-Abl*$^{-/-}$ senescent phenotype **(Fig. 3.2A)**. On the other hand, changes in $p16^{INK4a}$ expression was visibly noted, first an up-regulation with increasing passage (as cells senesce), and next, the apparent difference in mutant versus wild type osteoblasts **(Fig. 3.2A)**. It was noticed that with all western blot analyses, the levels of $p16^{INK4a}$ expression in wild type osteoblasts never achieved the maximal levels seen in *c-Abl*$^{-/-}$ at parallel passages, suggesting that up-regulation of $p16^{INK4a}$ may participate in the initiation and/or maintenance of senescence in osteoblasts.

More importantly, ectopic expression of $p16^{INK4a,}$ to a level comparable to that of *c-Abl*$^{-/-}$ osteoblasts, resulted in a senescent-like phenotype in early passage primary wild type osteoblasts; flattened cell morphology and two-fold reduction of BrdU incorporation **(Fig. 3.2B and Fig. 3.2C)**. To support the findings that $p16^{INK4a}$ up regulation contributes to osteoblast senescence, immunohistochemical staining of $p16^{INK4a}$ protein in the long bones of c-Abl adult mice was carried out. It revealed

higher levels of $p16^{INK4a}$ in c-Abl mutant mice, mostly concentrated at the growth plate region of the long bone, where active osteoblasts reside **(Fig. 3.2D)**. $p16^{INK4a}$ expression was also examined in the long bones of E18.5 embryos and they show a similar staining pattern between mutant and wild type, though to a lesser extent (results not shown). Extraction of RNA from the adult femurs also revealed higher $p16^{INK4a}$ mRNA levels in knockout **(Fig. 3.2E)**.

3.3 $p16^{INK4a}$ affects not only the senescence, but also regulates survival of *c-Abl*$^{-/-}$ osteoblasts

I have examined if the loss of $p16^{INK4a}$ in *c-Abl*$^{-/-}$ osteoblasts could revert the senescent phenotype. $p16^{INK4a}$ expression was knocked down in *c-Abl*$^{-/-}$ osteoblasts using p16shRNA (courtesy of Dr. S.Lowe) and its proliferation ability assessed by counting and replating every 4-5 days. It was observed that the viability of p16shRNA-*c-Abl*$^{-/-}$ osteoblasts could overcome the negative or neutral growth rate (at least 2 fold in twice repeated experiments) briefly over the 3^{rd}-4^{th} passage onwards, and ceased to divide thereafter (results not shown). This slight ability to overcome its usual senescing phenotype may indicate once $p16^{INK4a}$ –mediated senescence pathway is induced in osteoblasts, they become irreversibly resistant to $p16^{INK4a}$ silencing by $p16^{INK4a}$ shRNA, or that for this rescue phenotype to fully manifest it may require additional factors other than $p16^{INK4a}$ *per se*. Nevertheless, the role of $p16^{INK4a}$ in osteoblast survival *in vivo* was examined using TUNEL assay on c-Abl embryonic bone sections **(Fig 3.3)** and it revealed that *c-Abl*$^{-/-}$ bones contain more cells undergoing apoptosis at the growth plate region and further north, compared to wild type bones.

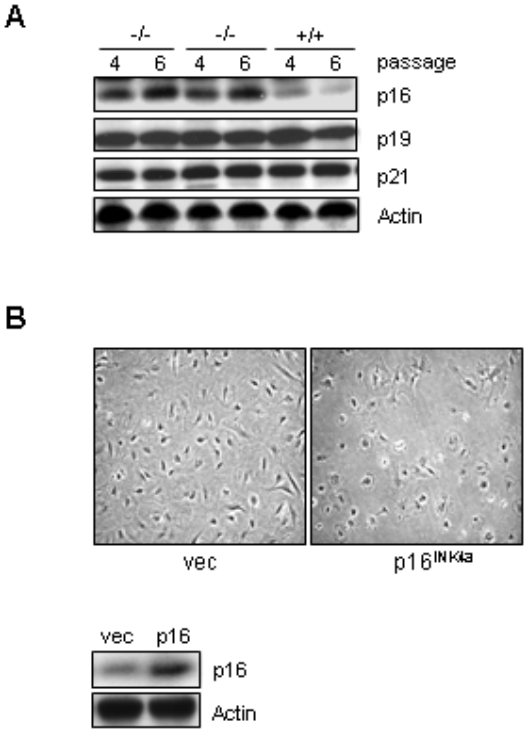
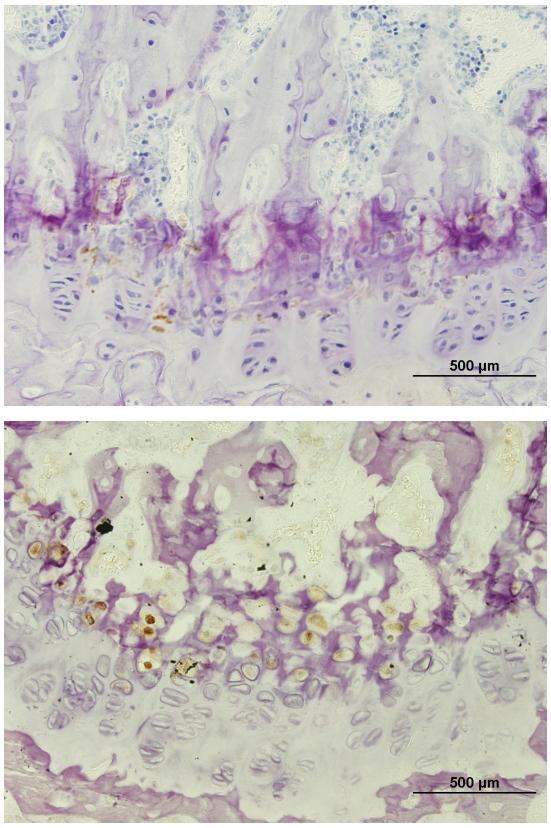

A
-/-
-/-
+/+
4
6
4
6
4
6
passage
p16
p19
p21
Actin
B
vec
p16INK4a
vec
p16
p16
Actin
C
BrdU labeling (artificial units)
0
1
2
3
4
5
*
p16
vec

Fig. 3.2. Up-regulation of $p16^{INK4a}$ expression in *c-Abl*$^{-/-}$ cells.

(A) *c-Abl*$^{-/-}$ osteoblasts expressed elevated levels of $p16^{INK4a}$, but not $p19^{ARF}$ or p21 during replicative senescence. Mutant and control osteoblasts were cultured as in Fig. 3.1, and at day 3 of indicated passage, cells were harvested and protein lysates normalized before western blot analysis. **(B)** Ectopic expression of $p16^{INK4a}$ led to senescence-like phenotypes. Wild type osteoblasts were infected with control empty retroviruses or viruses harboring $p16^{INK4a}$. Cells were selected against puromycin and cultured. Bottom panel: Western blot to show levels of infected $p16^{INK4a,}$ compared to control empty virus. **(C)** Forced expression of $p16^{INK4a}$ resulted in growth reduction, justified by a 2-fold reduction in S phase cells. The infected wild type osteoblasts were seeded in 96-well plates at various cell densities in triplicates and BrdU labeling was carried out overnight. Measurement of BrdU incorporation was determined by ELISA and averaged absolute units obtained. This experiment was repeated twice on separate occasions.



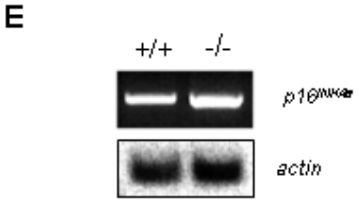


Fig. 3.2. Up-regulation of p16^{INK4a} expression in *c-Abl*$^{-/-}$ cells (continued).

(D) In vivo analysis of mouse bone sections. c-Abl adult mice femurs were decalcified and embedded in methyl methacrylate (MMA), sectioned and stained for p16^{INK4a} protein (40X magnification). (Top - *c-Abl*$^{+/+}$, Bottom - *c-Abl*$^{-/-}$). **(E)** mRNA levels of p16^{INK4a} examined in long bones of an c-Abl adult mice pair. Total RNA was extracted from femurs of an adult mice pair, and then subjected to RT-PCR.

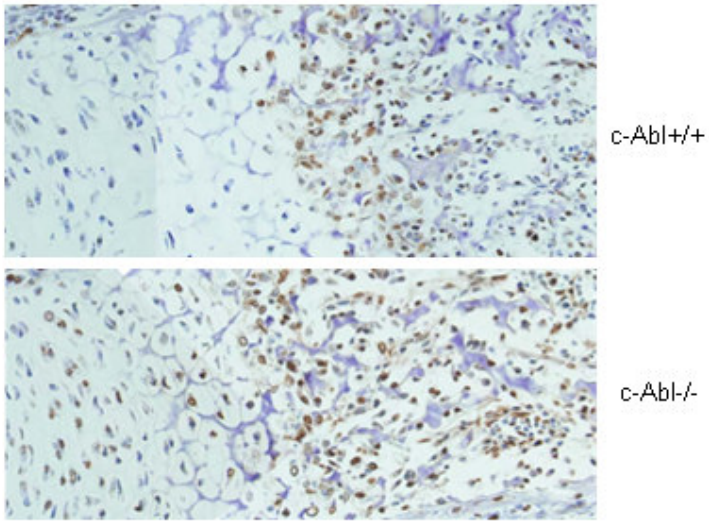


Fig. 3.3. Increased apoptosis in c-*Abl*$^{-/-}$ osteoblast long bones.

TUNEL assay immunostaining was performed on the long bones of c-Abl E18.5 day old embryos. Mice long bones were embedded in paraffin and sectioned (40X magnification).

3.4 Summary

Here, the osteoporotic phenotype of c-*Abl*$^{-/-}$ mice were further studied in detail at the cellular level. Primary cells were isolated from the cavarial bone to obtain relatively pure populations of osteoblasts for culture and biochemical assays. The osteoblasts used in this project were essentially unipotent osteoprogenitors, (or preosteoblasts, referred to as osteoblasts in this report) as they have the capacity to proliferate but do not express alkaline phosphatase or osteocalcein, markers for differentiated osteoblasts. However, in the presence of BMP2, or ascorbic acid and β-glycerol phosphate, they could differentiate into mature osteocytes.

Proliferation assay demonstrates a slower growth rate on c-*Abl*$^{-/-}$ osteoblasts (Fig 3.1A), and more advanced stage of senescence using the β-galactosidase marker (Fig 3.1B) with progressive passages when compared to wild type cells. These results complemented the *in vivo* data that c-*Abl*$^{-/-}$ mice show lower osteoblast numbers (Fig 3.1C), as well as significantly fewer cells in S phase in the long bones of c-*Abl*$^{-/-}$ embryos. Hence, the senile osteoporotic phenotype in c-*Abl*$^{-/-}$ mice stems from an innate problem with a reduced proliferation potential of its osteoblasts, apart from the defect in osteoblast function.

p16^{INK4a} was observed to be dramatically up-regulated in c-Abl deficient osteoblasts (Fig 3.2A). To confirm if p16^{INK4a} could mediate the senescence of osteoblasts, gain of function study on wild type osteoblasts revealed a senescent outcome, with cells displaying half the amount of cells in S phase, when compared to its control (Fig 3.2B and C). Hence, the stable p16^{INK4a} expression in c-*Abl*$^{-/-}$ osteoblasts could cause senescence, as without c-Abl, osteoblasts exhibit an advanced stage of senescence.

This outcome was exemplified by femur sections of adult mice, in which c-Abl mutant display increased staining for $p16^{INK4a}$, especially in the growth plate region, while RNA extracted from the long bones of adult mice pair expressed higher levels of $p16^{INK4a}$ transcript (Fig 3.2D and E).

Moreover, it has been shown that $p16^{INK4a}$ may be involved in regulating cell survival, in addition to senescence. Tunel assay performed on c-Abl E18.5 embryos showed increased apoptotic cells in c-Abl mutant long bones, suggesting that in the absence of c-Abl, higher $p16^{INK4a}$ expression correlates with increased senescence and apoptosis (Fig 3.3). Therefore c-Abl exerts a positive role in cell survival in osteoblasts, though it is not clear if the upstream regulators of $p16^{INK4a}$ may activate apoptotic/survival signaling pathways as well.

CHAPTER 4

UP-REGULATION OF $p16^{INK4a}$ IN *C-ABL*$^{-/-}$ OSTEOBLASTS IS ACCOMPANIED BY INCREASED ERK1/2 ACTIVATION AND DECREASED ID1 EXPRESSION

Chapter 3 describes $p16^{INK4a}$ as the candidate mediator of premature senescence in *c-Abl*$^{-/-}$ osteoblasts. The mechanisms behind regulation of $p16^{INK4a}$ in cellular senescence remains complicated with its heterogenous expression in different cell types or context. To date, some key transcription factors have been reported to regulate of $p16^{INK4a}$ expression. In $p16^{INK4a}$-pRB mediated senescence, Id1, E proteins and Ets proteins- Ets1 and Ets2 can modulate $p16^{INK4a}$ transcription.

Id1 delays senescence and was specifically involved in pRb, and not $p19^{ARF}$/p53-mediated senescence (Tang *et al*., 2002, Alani *et al*., 2001). Id1 was a negative regulator of $p16^{INK4a}$ by binding and sequestering the Ets1, which binds and activates the $p16^{INK4a}$ promoter. The reciprocal relationship of Id1 repression and Ets1 up-regulation was stable, and correlated with promoting $p16^{INK4a}$ expression (Ohtani *et al*. 2001). Similarly, the E protein–E47, a member of the HLH family, was also found to be antagonized by Id1 to block $p16^{INK4a}$ transcription (Zheng *et al*., 2004)

On the other hand, the MAPK cascade (Ras-Raf-MEK-ERK) was required for the up-regulation of $p16^{INK4a}$ and $p19^{ARF}$. Transcription factor Ets2, was found to be a downstream target of the MAPKs (Lin *et al*., 1998). Hence the primary activators of $p16^{INK4a}$ expression are the MAPKs, which activate Ets1/2, while Id1 serves to oppose

this. In this chapter the roles of MAPK-ERK and Id1 in *c-Abl*$^{-/-}$ osteoblasts are examined.

4.1 Role of MAPK pathway in the up-regulation of p16^{INK4a}

p16^{INK4a} can be positively regulated by the Ras-Raf-MAPK pathway, and consistent with this notion it was found that along with elevated p16^{INK4a} expression, *c-Abl*$^{-/-}$ osteoblasts also exhibited higher levels of activated ERKs (phospho-p42/p44) **(Fig. 4.1A)**. The overnight osteoblast cultures displayed intensified levels of activated ERK1/2, compared to cells cultured for 3 days (refer to passage "-" and "4"), probably due to the presence of fresh growth factors in the serum. Yet activated ERK1/2 remained higher in c-Abl mutant osteoblasts than in wild type cells, at all passage points **(Fig. 4.1A)**. This reflected the p16^{INK4a} expression profile between the both cell types.

Furthermore, specific inhibition of ERK using MEK inhibitor U0126 also reduced p16^{INK4a} levels in both osteoblast types, validating that ERK activation have a positive impact on p16^{INK4a} expression **(Fig. 4.1B)**. The fact that ERK inhibition in c-Abl mutant did not reduce the levels of p16^{INK4a} expression to that of wild type osteoblasts suggests that p16^{INK4a} up-regulation may involve other factors apart from ERK1/2, and possibly the inhibition of MEK (U0126) may have displayed slight redundancy by affecting the regulation of other proteins necessary for p16^{INK4a} regulation in the absence of ERK. Nonetheless, deficiency of c-Abl could alter ERK activation and this was affirmed by enhanced ERK activation upon inhibition of c-Abl using imatinib mesylate (**Fig. 4.1C**), and reconstitution of c-Abl in *c-Abl*$^{-/-}$ osteoblasts could repress

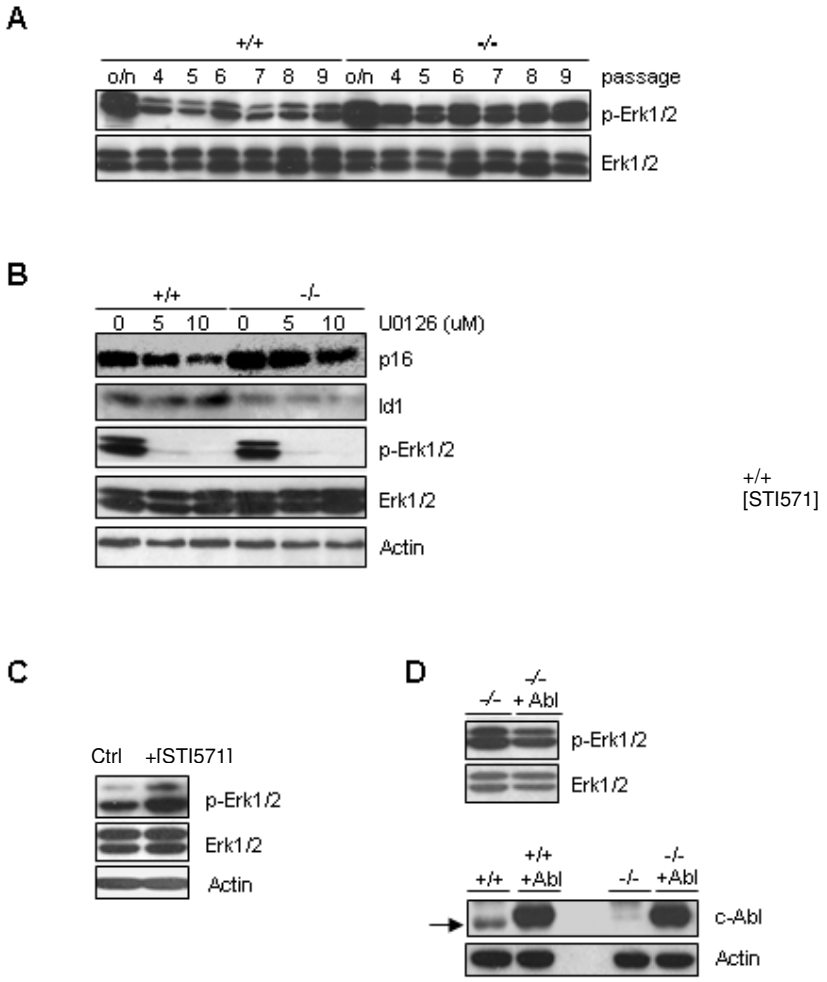


Fig. 4.1. Enhanced activation of ERK1/2 in *c-Abl*$^{-/-}$ osteoblasts.

(A) Mutant and wild type cells were cultured as in Fig. 3.1 and cells collected at day 3 of each passage. Passage "n/o" stands for cells that were grown overnight. Harvested lysates were subjected to western blot analysis. **(B)** Inhibition of ERK1/2 activation repressed p16^{INK4a} expression. Early passage (P2) osteoblasts were treated with 2 different doses of U0126 for 3 days and harvested for western blot (lanes 1 and 4 as non-treatment control). **(C)** c-Abl inhibition enhances ERK activation. Wild type osteoblasts (ctrl) were serum starved overnight and then treated with 1uM of STI571/imatinib for 2 hours. **(D)** Reconstitution of c-Abl in *c-Abl*$^{-/-}$ osteoblasts restores the normal activation of ERKs. Bottom panel: protein levels of retroviral expressed c-Abl in wild type and mutant osteoblasts.

ERK activation **(Fig. 4.1D)**. Taken together, these results summarize c-Abl negative effect on ERK activation and will be further discussed in Chapter 7.

4.2 An opposing role of Id1 in p16^{INK4a} up-regulation

The expression of p16^{INK4a} is largely controlled by the helix-loop-helix family of transcription factors, namely the Ets1/2 as positive regulators and Id1 as a negative regulator (Ohtani *et al*., 2001). Besides, Id1 involvement in p16^{INK4a}-dependent senescence have been well studied (reviewed in Zebeedee and Hara, 2001, Ruzinova and Benezra, 2003), which raised the rationale to probe the expression of these proteins in *c-Abl*$^{-/-}$ osteoblasts. While the expression of Ets1 and Ets2 remained unchanged (data not shown), the levels of Id1 protein was down-regulated in *c-Abl*$^{-/-}$ osteoblasts **(Fig. 4.2)**. Since Id1 levels did not change with increasing passages, it was possible that the constant p16^{INK4a} up-regulation during osteoblast senescence could be triggered by other factors other than Id1. Nevertheless, an inverse relationship could be observed in untreated osteoblasts; when p16^{INK4a} expression was increased, Id1 level dropped **(Fig. 4.1B,** lanes 1 and 4**)**. This expression pattern was also observed in long bone sections of c-Abl fetuses (E18.5), where Id1 immunostaining is lower in c-Abl mutants compared to wild type (results not shown).

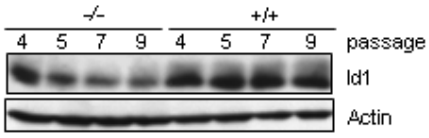


Fig. 4.2. Down-regulation of Id1 in *c-Abl*$^{-/-}$ osteoblasts.

The osteoblasts were harvested at day 3 following the designated passage number and probed with western blot antibodies for Id1 and β-actin.

4.3 Summary

The increase in $p16^{INK4a}$ expression in senescent osteoblasts correlates with aging-associated phenotypes such as increased β-Gal staining and a reduction in BrdU positive cells. What are the upstream mediators that regulate $p16^{INK4a}$ expression in *c-Abl*$^{-/-}$ osteoblasts? The endogenous levels of activated MAPK-ERK1/2 was found to be dramatically elevated in *c-Abl*$^{-/-}$ osteoblasts, when compared to wild type cells, and this increase remained consistently unchanged, regardless of osteoblast passage number (Fig. 4.1A). In addition, the presence of c-Abl displays an inhibitory mode on ERK activation, as blocking c-Abl kinase activity using imatinib/STI571 relieved the ERK inhibition in wild type osteoblasts (Fig. 4.1C), while ERK repression was restored in *c-Abl*$^{-/-}$ osteoblasts, when c-Abl was reinstated (Fig. 4.1D).

On the other hand, as Id1 has been shown to inhibit $p16^{INK4a}$ expression in the $p16^{INK4a}$-RB mediated senescent pathway. Aligned to our results, Id1 expression was diminished in *c-Abl*$^{-/-}$ osteoblasts and at the *in vivo* level, in the long bones of *c-Abl*$^{-/-}$ embryos (Fig 4.2 and results not shown). As a result the lack of sufficient Id1 levels in c-Abl mutants is likely to be one of the reasons why $p16^{INK4a}$ was higher in the mutants than in wild type osteoblasts.

Hence, the positive impact of increased ERK activation and the negative influence of Id1 exemplify the actions of two opposing mediators that must be balanced in order to achieve a desired output of $p16^{INK4a}$ on osteoblasts. Yet, $p16^{INK4a}$ expression in c-Abl osteoblasts suggests the existence of regulation by separate pathways; one that

involve an ERK1/2 mediated pathway independent of Id1, and another pathway that requires Id1 (Fig 4.1B).

CHAPTER 5

C-ABL POSITIVELY REGULATES ID1, VIA THE BMP-SMADS-1/5/8 SIGNALING PATHWAY

Previous chapters have demonstrated the strong correlation of Id1 up-regulation with $p16^{INK4a}$ down-regulation in the premature senescence of osteoblasts (depicted in chapter 3). This chapter further investigates the upstream regulation of Id1 and its relationship with the BMP pathway, to understand how Id1 expression is affected by c-Abl deficiency during osteoblast senescence.

5.1 c-Abl is likely to augment Id1 expression via the BMP pathway

Having shown that *c-Abl*$^{-/-}$ osteoblasts expressed reduced levels of Id1, and Id1 is a direct target gene of the BMP pathway (Miyazono and Miyazawa, 2002), it was imperative to understand how c-Abl regulates Id1 expression. More so as it is known that BMPs are expressed and secreted by osteoblasts, and BMP signaling is essential for regulating osteoblast differentiation and bone remodeling (Chen *et al*., 2004). To demonstrate that c-Abl is involved in BMP2-induced Id1 expression, *c-Abl*$^{-/-}$ and control osteoblasts were serum starved overnight and stimulated with increasing doses of BMP2 **(Fig. 5.1A)**. Both cells were highly sensitive to BMP2, with dramatic Id1 induction even at a low dose of 10ng/ml BMP2, while the Id1 levels in mutants remained sub-optimal, and did not reach that of wild type osteoblasts. Surprisingly, BMP2 at the dose of 200ng/ml failed to induce Id1, probably due to feedback regulation in the cell response to high concentrations of BMP2. In addition, inhibition of c-Abl using imatinib was able to repress BMP2-induced Id1 expression in wild

type osteoblasts **(Fig. 5.1B)**, indicating c-Abl was required for maximal induction of Id1.

To test if c-Abl reconstitution could ultimately rescue the defect of BMP-induced Id1 expression in *c-Abl*$^{-/-}$ osteoblasts; retroviruses carrying the c-Abl gene and empty vector as control were infected into these cells and then challenged with 10ng/ml of BMP2. The time course study showed that c-Abl could indeed rescue the otherwise weak Id1 induction in *c-Abl*$^{-/-}$ osteoblasts, when compared to the vector control in the same set of cells, even with a low dose of BMP2 **(Fig 5.1C)**. This implies c-Abl plays a positive role in BMP signaling, while negatively regulating ERK1/2 activation (Fig. 4.1D).

5.2 c-Abl up-regulates Id1 transcription

To explore the extent of c-Abl regulation of the Id1 gene, transcription-based assays were carried out. In RT-PCR assays, overexpression of c-Abl or the constitutively activated v-Abl DNA enhanced the endogenous Id1 mRNA expression, with stronger induction being seen with v-Abl in osteoblast-like cell line MC3T3 **(Fig. 5.2A)**.

It was shown that c-Abl can potentiate BMP-induced Id1 at the protein and transcription levels (Fig 5.1C, 5.2A). Luciferase reporter assays using Id1 promoter construct were transfected into myoblastic cell line C2C12. Constructs expressing effectors of BMPs; Smad1 and Smad4 with BMP2, or BMP2 alone was able to enhance Id1 transcription, yet c-Abl itself could only induce a 2-fold increase in basal Id1 transcription. The kinase activity of Abl was important for this, as constitutive activated Abl, v-Abl, induced significant promoter activity while addition of the

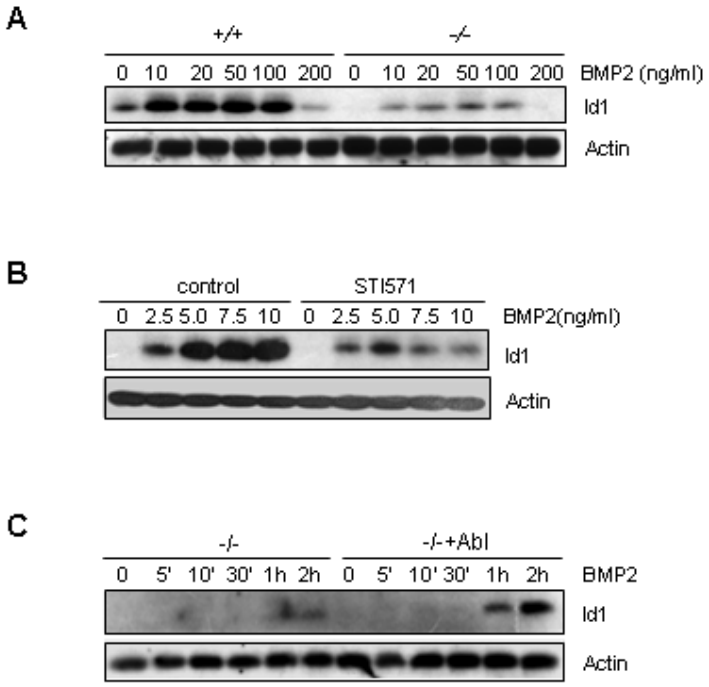


Fig. 5.1. c-Abl is required for optimal induction of Id1.

(A) Reduced Id1 induction by BMP2 in *c-Abl*$^{-/-}$ osteoblasts. c-Abl mutant and wild type osteoblasts were serum-starved overnight and stimulated with various doses of BMP2 for 4 hrs and the levels of Id1 determined by Western blot. **(B)** Imatinib treatment inhibits Id1 induction by BMP2. Primary osteoblasts were pre-treated with 1uM imatinib for an hour prior to adding of low doses of BMP2 for 1 hr. **(C)** A time course study of Id1 induction in *c-Abl*$^{-/-}$ and c-Abl reconstituted *c-Abl*$^{-/-}$ osteoblasts using 5ng/ml of BMP2.

kinase-dead c-Abl [KD] remained unchanged **(Fig. 5.2B)**, this also signifies the effects of v-Abl and the BMP-Smad pathway is not mutually exclusive. Treatment of C2C12 cells transfected with Id1 promoter-luciferase gene with imatinib to inhibit endogenous c-Abl also repressed Id1 transcription, as well as BMP-induced Id1 transcription **(Fig. 5.2C)**, stressing the need for c-Abl kinase activity in Id1 up regulation, whether direct or indirect.

Importantly, RT-PCR assay confirmed that c-Abl regulates Id1 at the transcript level as the reconstitution of c-Abl into *c-Abl*$^{-/-}$ osteoblasts could rescue the BMP-induced Id1 mRNA **(Fig. 5.2D)**, modeling the Id1 protein level pattern observed in Fig.5.1C.

5.3 c-Abl enhances Smad activation to promote expression of BMP target genes

Since c-Abl positively regulates Id1 gene transcription, and more so with BMP-induced Id1, It was necessary to determine if BMP-Smads 1/5/8 could be affected by c-Abl deficiency, since Smads1 and Smad4 are the upstream regulators of Id1 (Lopez-Rovira *et al*., 2002), and/or if this involved a Smad-independent mechanism. *c-Abl*$^{-/-}$ osteoblasts were subjected to BMP2 in various doses, revealing compromised Smad 1/5/8 activation, as well as basal levels when compared to wild type cells **(Fig. 5.3A)**. Also, while the basal levels of activated Smad 1/5/8 was significantly reduced in mutant cells, they could be restored by the reconstitution of c-Abl **(Fig. 5.3B)**. Both suggest the compromised Smad signaling could be responsible for the down regulation of Id1. This was not confined solely to osteoblasts, as *c-Abl*$^{-/-}$ MEFs also displayed such a response to BMP2, along with up-regulation of ERK activation **(Fig. 5.3C**).

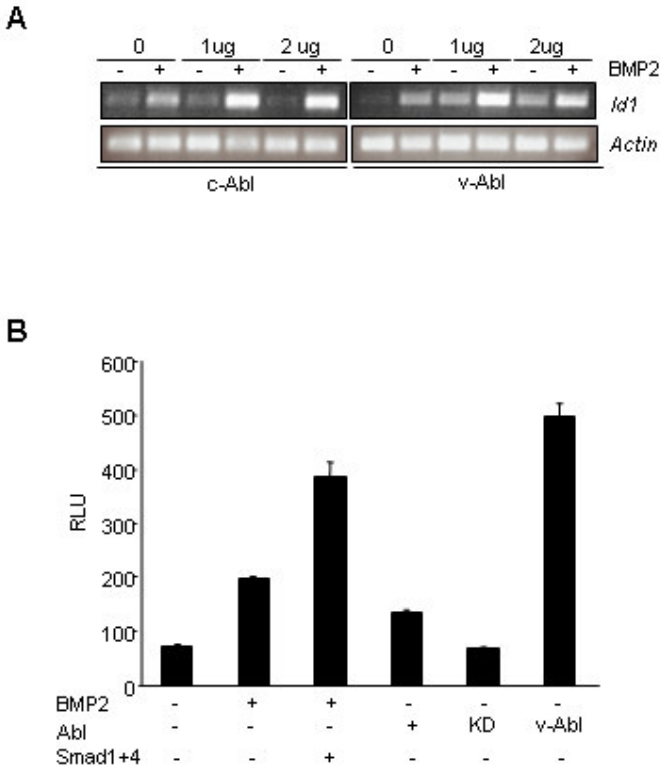


Fig. 5.2. c-Abl regulates Id1 at the level of transcription.

(A) c-Abl overexpression promoted BMP-induced Id1 expression. MC3T3 cells were transfected for 24 hrs, and further treated with 100ng/ml of BMP2 for 6 hrs. The amount of Id1 mRNA was determined by RT-PCR. **(B)** c-Abl acted directly on the Id1 promoter. Id1 promoter-luciferase reporter was co-transfected with Smad1 and Smad4, c-Abl, c-Abl kinase dead [KD], or v-Abl into C2C12 cells for 3.5 hrs. These cells were then treated with 100ng/ml BMP2 overnight for 16 hrs and the luciferase activity was measured with the renilla plasmid as an internal control.

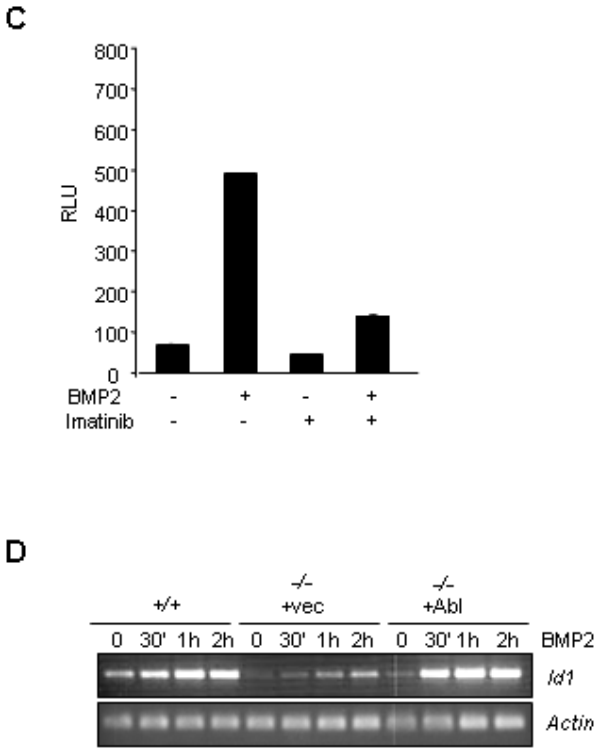


Fig. 5.2. c-Abl regulates Id1 at the level of transcription (continued).

(C) Imatinib mesylate inhibited Id1 promoter activity. As before, Id1 promoter-luciferase reporter was transfected into C2C12 cells. The cells were then pretreated with 1uM imatinib for 2 hrs prior to adding 100ng/ml BMP2 for 16 hrs. Luciferase activity was measured, and renilla used as an internal control. **(D)** RT-PCR assay showed that BMP2-induced Id1 mRNA was significantly reduced in *c-Abl*$^{-/-}$ osteoblasts and can be rescued upon c-Abl reconstitution using retrovirus. c-Abl mutant and control osteoblasts were stimulated with 5ng/ml BMP2 and harvested at various time points, and assessed for the level of Id1 mRNA.

As the activation of Smads 1/5/8 would result in changes to downstream events of BMP signaling since they mediate the transcription of various target genes, RT-PCR was carried out in the same conditions as in Fig 5.2A to study the mRNA levels of other target genes. BMP early target genes such as Id3 and Smad6 gave rise to similar profiles as Id1 transcript, whereby attenuated response to BMP2 exist with the lack of c-Abl, and this deficiency could be restored with c-Abl reconstitution **(Fig 5.3D)**. These results reaffirms the hypothesis that c-Abl acted positively on the BMP pathway and its direct effector Smads 1/5/8, and is needed for full induction of BMP target genes.

.

5.4 BMP sustains Id1 expression to promote cell proliferation

To place in context the logic behind Id1 up-regulation by c-Abl in the senesence of osteoblasts, the Id1 protein was overexpressed in *c-Abl*$^{-/-}$ osteoblasts using retrovirus **(Fig. 5.4A)**. Levels of Id1 overexpressed in mutant cells matched that of wild type levels and was sufficient to down regulate $p16^{INK4a}$, and extented the lifespan of mutant cells by three doublings (results not shown). This establishes the basis that c-Abl elevation of Id1 served to down regulate $p16^{INK4a}$, and delay the senescent fate of osteoblasts, and is consistent with findings that Id1 overexpression extended the lifespan of keratinocytes and ES cells (Rheinwald *et al*., 2002, Ying *et al*., 2003).

In order to prove BMP signaling affects $p16^{INK4a}$ expression via Id1, BMP2 and BMP4 antagonists: noggin and chordin, were added to culture media of wild type osteoblasts. While activated Smads 1/5/8 and target gene Id1 were repressed, $p16^{INK4a}$

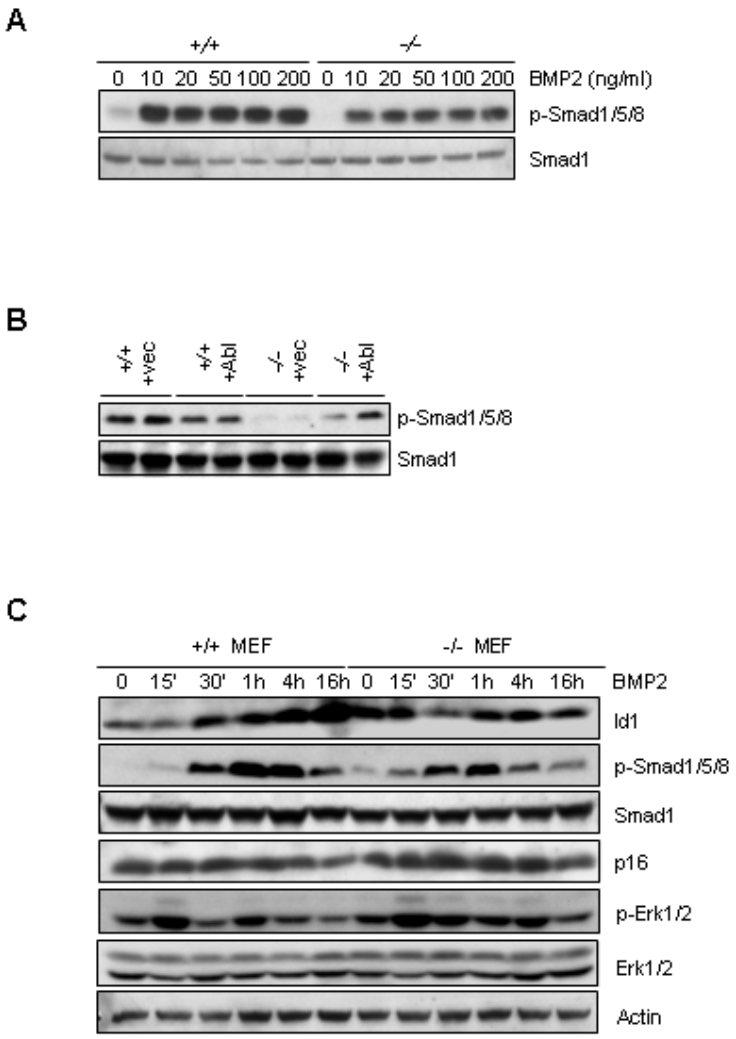


Fig. 5.3. c-Abl regulates the activation of BMP-Smad1/5/8.

(A) Reduced Smad1/5/8 activation in the presence of BMP2. *c-Abl*$^{-/-}$ and control osteoblasts were stimulated with various concentrations of BMP2 for 4 hrs and analyzed by western blot using anti-p-Smad1/5/8 antibodies. **(B)** The attenuated basal levels of activated Smads in *c-Abl*$^{-/-}$ osteoblasts were restored by c-Abl reconstitution. **(C)** Profile of Id1, Smad1/5/8, p16^{INK4a} and ERKs expression in response to BMP2 in c-Abl MEFs is similar to that of osteoblasts. MEFs isolated from *c-Abl*$^{-/-}$ mice and control littermates were cultured to passage 3. Cells were treated with BMP2 for different time points and harvested for western blot analyses.

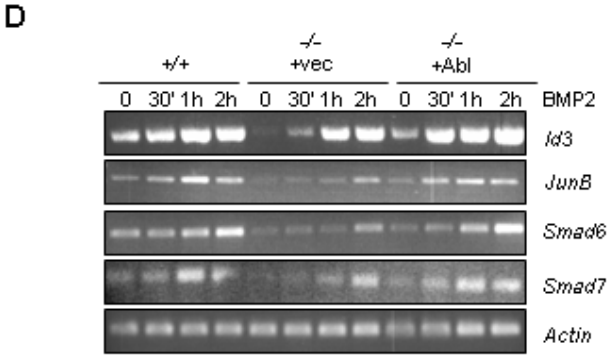


Fig. 5.3. c-Abl positively regulates BMP signaling via Smad1/5/8 activation.

(D) RT-PCR assays conducted in the same manner as in Fig. 5.2D also revealed the loss of BMP2 induction on BMP target genes, and can be rescued to wild type levels with the addition of c-Abl back to *c-Abl*$^{-/-}$ osteoblasts. 5ng/ml BMP2 was added for various time points before subjecting cells to RNA extraction and RT-PCR.

expression was concurrently elevated with increasing dosage of BMP antagonists **(Fig. 5.4B)**. Moreover, BMP2 was able to down regulate $p16^{INK4a}$ in osteoblast cultures **(Fig. 5.4C)**, also reflected in c-Abl MEFs (Fig. 5.3C), stressing the importance of an intact BMP pathway in influencing $p16^{INK4a}$ expression. Nonetheless, the delayed rate of $p16^{INK4a}$ up regulation (Fig. 5.4B), and resistance to absolute repression of $p16^{INK4a}$ (Fig. 5.4A and C) also meant that the regulation of $p16^{INK4a}$ gene is complex and likely to involve a combination of other factors for complete regulation.

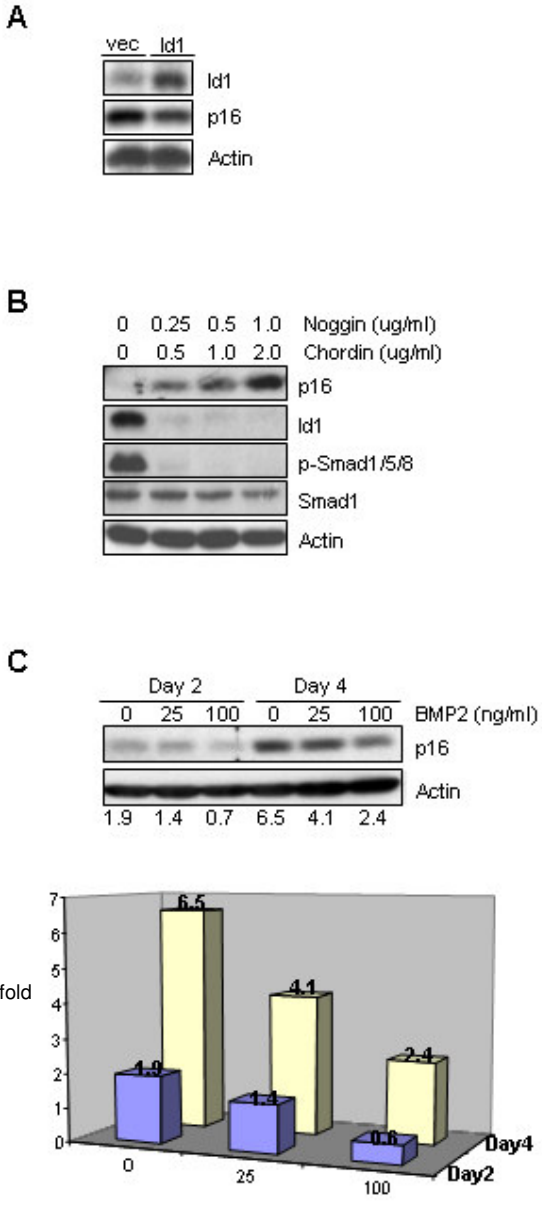

A
vec
Id1
Id1
p16
Actin
B
0 0.25 0.5 1.0 Noggin (ug/ml)
0 0.5 1.0 2.0 Chordin (ug/ml)
p16
Id1
p-Smad1/5/8
Smad1
Actin
C
Day 2
Day 4
0 25 100 0 25 100 BMP2 (ng/ml)
p16
Actin
1.9 1.4 0.7 6.5 4.1 2.4
p16INK4a fold increase
6.5
4.1
2.4
1.9
1.4
0.6
Day4
Day2
0
25
100

Fig. 5.4. BMP2-induced Id1 up-regulation promotes osteoblast proliferation.
(A) Retroviral expression of Id1 down-regulated $p16^{INK4a}$ in *c-Abl*$^{-/-}$ osteoblasts. **(B)** Blocking BMP signaling with noggin and chordin in osteoblasts down-regulates Id1 and up-regulates $p16^{INK4a}$. Wild type osteoblasts were cultured in the presence of increasing doses of noggin and chordin for 3 days and the levels of $p16^{INK4a}$, Id1 and phospho-Smad1/5/8 determined by western blot. **(C)** BMP2 was able to down-regulate $p16^{INK4a}$ expression in osteoblast cultures. Normal osteoblasts were seeded at $1x10^5$ cells/60mm dish, and treated with BMP2 for 2 or 4 days. The basal levels of $p16^{INK4a}$ were determined by western blot. Graphical representation of normalized $p16^{INK4a}$ levels (against actin) using densitometry.

5.5 Summary

In c-Abl wild type osteoblasts, the homeostatic levels of $p16^{INK4a}$ is maintained by Id1 and counter-balanced by ERKs to achieve a normal replicative senescence program reflective of primary cells. The reduction of Id1 expression in c-Abl mutant cells prompted further investigation of its role not only in affecting $p16^{INK4a}$ mediated senescence, but also in the search for a connection between c-Abl and Id1. c-Abl was found to positively regulate Id1 expression, as seen when comparing the basal Id1 protein expression levels, as well as under BMP2 induction , where the difference was clearly observed under conditions of serum starvation (Fig 5.1A). In addition, inhibition of c-Abl on a separate batch of wild type osteoblasts shows that Id1 expression is augmented by c-Abl under low doses of BMP2. However, as serum-free media was used for imatinib pretreatment period, as well as on non-treated osteoblasts, and the density of protein cell lysate collected was lesser in comparison to other sets, this could account for the minimal Id1 expression seen in lane1 of control set cells (Fig 5.1B). Conversely, the rescue of BMP2-induced Id1 in c-Abl knockout osteoblasts is restored with reconstitution of c-Abl (Fig 5.1C). Therefore this suggested that c-Abl could be involved in BMP signaling and affect its target gene Id1. Furthermore, transient overexpression of v-Abl could significantly boost basal Id1 mRNA, while the addition of BMP2 saturates this effect by the maximal induction of Id1 in MC3T3-E1 cells. This occurred to a lesser extent in the c-Abl overexpressed set, probably due to the difficulty of getting sufficient c-Abl kinase activation to occur in these cells (Fig 5.2A). c-Abl also appears to create an additive effect on Id1 transcription, and the degree of Id1 induction depended on its kinase action as the kinase dead c-Abl, as well as inhibition of Abl kinase by imatinib, repressed Id1 transcription, demonstrated by the Id1-promoter reporter assays (Fig 5.2B and C).

Again, the rescue of endogenous Id1 and BMP-induced Id1 mRNA in *c-Abl*$^{-/-}$ osteoblasts by c-Abl reconstitution (Fig 5.2D) was consistent with the pattern seen at the protein level (Fig 5.1C). The rapid induction of BMP2-induced Id1 by c-Abl led to the analysis of BMP-Smads expression, and indeed the activation of Smads1/5/8 was compromised by the loss of c-Abl, particularly at the endogenous stage. This phenomenon was not restricted to osteoblasts as *c-Abl*$^{-/-}$ MEFs also displayed poorer BMP2-induced Id1 expression and Smad activation. Smads are transcription factors that regulate a large repertoire of genes, hence this led to extending the analysis of c-Abl reconstitution studies to more BMP target genes. Undoubtedly, the transcription of various immediate-early direct target genes of the BMP pathway were found to be affected (Fig 5.3). This led to the conclusion that c-Abl could participate in the BMP signaling pathway, and do so in a positive manner.

To confirm if c-Abl's involvement in BMP pathway could modulate p16^{INK4a} mediated senescence in osteoblasts, Id1 overexpression in *c-Abl*$^{-/-}$ osteoblasts could down-regulate p16^{INK4a} expression and overcome the premature senescence to some extent (Fig 5.4A). In addition, BMP2 could repress p16^{INK4a} expression further in proliferating osteoblasts with increasing dosage (Fig 5.4C), while blocking BMP signaling and feedback by BMP antagonists created an outcome that supported rising evidence for BMP regulation of p16^{INK4a}, as it appears the repressive effect of BMP on p16^{INK4a} was lifted, in a dose dependent manner, and correlated with repression of Id1 and Smad activation. Thus, c-Abl could augment BMP2-induced Id1 to promote osteoblast proliferation, in tandem with the function of Id1 up-regulation to maintain p16^{INK4a} repression.

CHAPTER 6

C-ABL ACTS UPSTREAM OF SMADS-1/5/8 AT THE BMP RECEPTOR LEVEL TO AUGMENT BMP-SMAD SIGNALING

The structural features of BMP receptors were described in chapter 1.4.1 (page 37). Smads1/5/8 can only be activated by type I BMPRs (BMPRIA), therefore BMPRIA determines the specificity of Smad signaling. Proper BMP-Smad signaling requires intact type I BMP receptors, as studies manipulating BMPRIA have shown. Truncated forms of BMPRIA caused cells to differentiate into myotubes (Namiki *et al*., 1997), and induced dorsal mesoderm formation instead of ventralization in *Xenopus* embryos (Suzuki *et al*, 1994). So, mutated BMPRIA appears to exert a dominant negative effect in vitro and in vivo, since the phenotype completely reversed.

In this chapter the roles of BMP receptors are covered, to gain an understading into the attenuated Smad activation and Id1 induction in *c-Abl*$^{-/-}$ osteoblasts.

6.1 c-Abl phosphorylates BMP type I receptors

The endogenous transcript and protein levels of BMP receptors 1A, 1B and II were examined in c-Abl wild type and knockout osteoblasts and MEFs. No obvious changes were observed, which ruled out varying innate expression levels as the reason behind decreased Smad activation in *c-Abl*$^{-/-}$ osteoblasts. **(Fig. 6.1A; transcript in osteoblasts, protein in MEFs)**. To determine the likelihood of post-translational modification of the receptors, c-Abl was cotransfected along with receptor 1A, 1B or II in cos7 cells, and subjected to immunoprecipitation. It was observed that tyrosine

phosphorylation occurred in type 1 receptors and not type II when co-expressed with c-Abl **(Fig. 6.1B)**. Moreover, since no tyrosine phosphorylation could be seen with co-expression of a kinase dead version of c-Abl (KD), this indicated a requirement for cAbl activation in this process (lane 6 in Fig 6.1B). To test whether this modification was physiological, *c-Abl*$^{-/-}$*Arg*$^{-/-}$ double knockout (DKO) MEFs were used to immunoprecipitate endogenous BMPR1A. While similar levels of BMPR1A could be immunoprecipitated in both DKO and WT MEFs, DKO MEFs displayed lower levels of tyrosine phosphorylation compared to wild type **(Fig. 6.1C)**. In addition, the compromised BMPR1A tyrosine phosphorylation in DKO MEFs was also seen in *c-Abl*$^{-/-}$ MEFs, and this effect was further enhanced with BMP induction **(Fig. 6.1D).** It is possible that the homologue of c-Abl; Arg, might be needed to exert some synergistic kinase activity on c-Abl, to influence the degree of BMPR1A tyrosine phosphorylation.

6.2 Serial deletion analysis of BMPR1A reveals the carboxy terminal region is phosphorylated by c-Abl

The intracellular portion of BMPR1A consists mainly of a ~32 kDa kinase domain, leaving a short tail of 13 aa at its carboxy terminus. In order to identify the region where c-Abl phosphorylates, deletion analysis was performed. 4 fragments with the lengths of 50 kDa, 45 kDa, 35 kDa or 30 kDa, with truncations starting from the carboxy terminus were PCR-cloned using the full length (FL) wild type BMPR1A as template, and HA-tagged. These deletions were labeled as deletion 1-4 (1 being the shortest fragment – 30 kDa), with each fragment consisting of a cluster of tyrosine residues (illustrated in **Fig. 6.2A**). Along with FL receptor, these constructs were co-

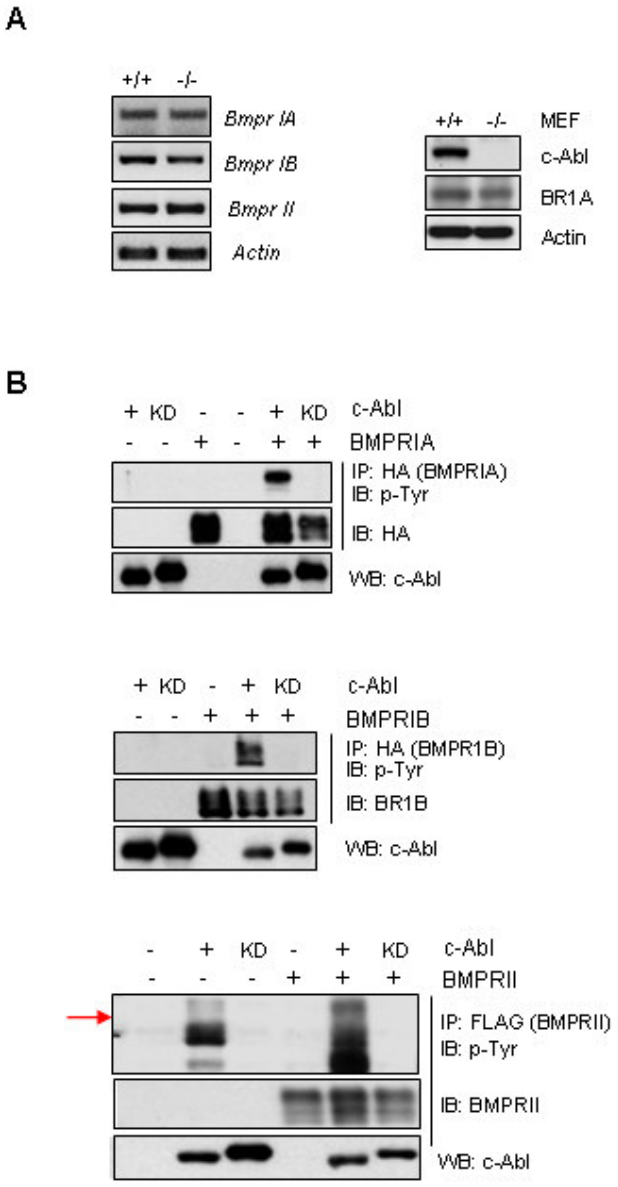

A
+/+ -/-
Bmpr IA
Bmpr IB
Bmpr II
Actin
+/+ -/-
MEF
c-Abl
BR1A
Actin
B
+ KD - - + KD
c-Abl
- - + - + +
BMPRIA
IP: HA (BMPRIA)
IB: p-Tyr
IB: HA
WB: c-Abl
+ KD - + KD
c-Abl
- - + + +
BMPRIB
IP: HA (BMPR1B)
IB: p-Tyr
IB: BR1B
WB: c-Abl
- + KD - + KD
c-Abl
- - - + + +
BMPRII
IP: FLAG (BMPRII)
IB: p-Tyr
IB: BMPRII
WB: c-Abl

Fig. 6.1. Post-translational modification of BMP type I receptors by c-Abl.

(A) Left - c-Abl mutant and wild type osteoblasts were harvested and subjected to RNA extraction and then RT-PCR. Little difference was observed in mRNA expression levels of BMPR 1A, 1B and II. Right – Protein levels of BMPR1A in c-Abl MEFs. **(B)** Co-expression with c-Abl led to BMPR1A and BMPR1B tyrosine phosphorylation. HA-tagged BMPR1A and BMPRIB was expressed alone or co-expressed with c-Abl in cos7 cells. BMP receptor was immunoprecipitated using anti-HA antibodies and its tyrosine phosphorylation determined by western blot. Similarly, Flag-tagged BMPRII was immunoprecipitated with anti-Flag antibodies and observed for tyrosine phosphorylation.

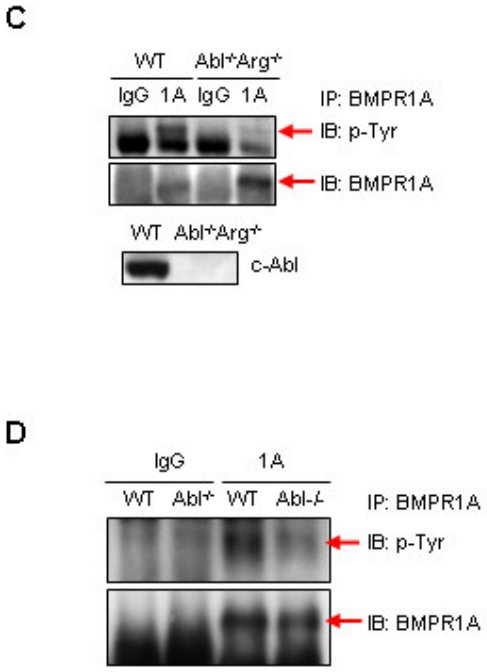


Fig. 6.1. Tyrosine phosphorylation of BMP type I receptors by c-Abl (cont'd).
(C) Reduced tyrosine phosphorylation of BMPR1A in *c-Abl*$^{-/-}$*Arg*$^{-/-}$ MEFs. Endogenous BMPR1A was immunoprecipitated from cell lysates of mutant and control MEFs and tyrosine phosphorylation determined by western blot. **(D)** *c-Abl*$^{-/-}$ and wild type MEFs were grown to confluence, serum starved overnight and 15ng/ml BMP2 added the next day for 2.5 hours before harvesting cells. Cell lysates were subjected to similar conditions as in (C) during immunoprecipitation.

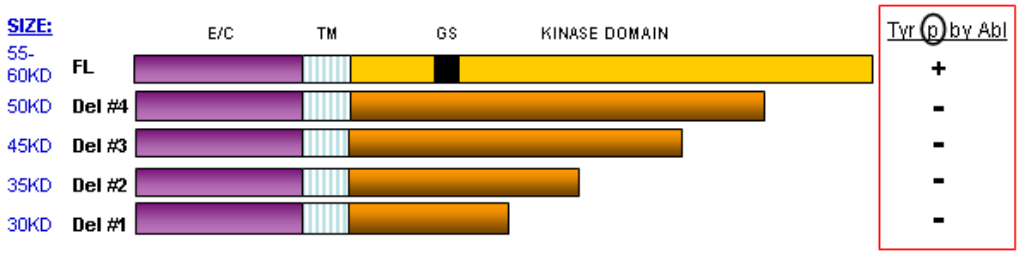
expressed with c-Abl in cos7 cells and upon immunoprecitation of receptor via HA antibody, no tyrosine phosphorylation could be seen in all 4 deletion constructs except for the original FL receptor (results summarized in **Fig. 6.2A** right panel box). This leads to the finding that the tail-end region or carboxy terminus of BMPR1A is important in c-Abl regulation.

6.3 BMPR1A Y453/467 residues are preferentially phosphorylated by Abl kinases

In order to map the tyrosine residues in the tail-end region, an alignment of BMPR1A protein sequences of various animal species was made **(Fig. 6.2B)**. 17 conserved tyrosine residues could be found in the entire cytoplasmic length of BMPR1A, with a cluster of 4 tyrosine residues being found at the extreme C-terminus, designated Y453, Y457, Y458 and Y467 **(Fig. 6.2B).** Mutagenesis of these four tyrosine residues were performed to generate four single tyrosine (Y) FL receptor mutants (labeled A to E) by replacing its original tyrosine residue to phenylalanine (F), so it does not affect protein confirmation, yet fail to be phophorylated by tyrosine kinases. Tyrosine phosphorylation by immunoprecipation was examined with these 4 single Y receptor mutants and no difference in the ability or strength for c-Abl phosphorylation was found **(Fig. 6.3A)**. This led us to speculate that these Y residues might work in a group rather than singly to affect c-Abl action on BMPRs. Subsequently, 4 more tyrosine mutants were made with the first four mutants as templates, this time in various Y residue combinations as illustrated in **Fig. 6.3B**. Phosphorylation analysis of these 4 combination mutants show that relative to FL wild type receptor, the tyrosine phosphorylation by c-Abl was abolished in the mutant I **(Fig. 6.3B).**

Moreover, the addition of BMP could enhance the c-Abl tyrosine phosphorylation of combination receptor mutants F, G, and H, but could not lift the severe inhibition in mutant I **(Fig. 6.3C)**. The level of phosphorylation drops with receptor mutant H, thus suggesting that residues Y453 and Y467 are the tyrosine residues preferred by c-Abl. In addition, the degree of tyrosine phosphorylation by c-Abl is possibly quantity dependent, as receptor mutant F displayed highest phosphorylation, corresponding to higher c-Abl expression when compared to the rest. This overall effect was enhanced when the mutants were co-expressed with constitutive v-Abl (figure not shown), hence confirming that the integrity of Abl phosphorylation of BMPR1A depends largely on the combination of Y453 and Y467.

A



B

BMPR1A alignment

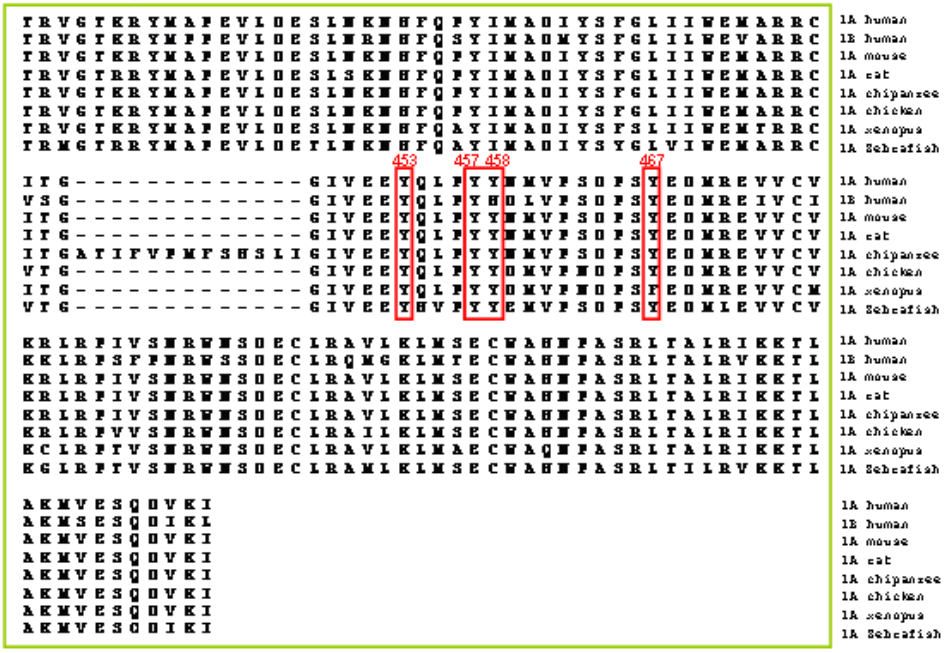
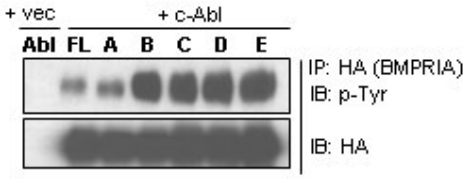


Fig. 6.2. Mapping of BMPR1A phosphorylation site by c-Abl via receptor truncations.

(A) The domain phosphorylated by c-Abl was mapped by serial deletions starting from C-terminus end. Four deletion mutants were created with their respective sizes stated on the left, compared to full length [FL] 1A receptor. Receptor deletion mutants #1 to 4 were co-expressed with c-Abl and tyrosine phosphorylation determined by western blot. The box in red summarizes the lack of tyrosine phosphorylation observed in all deletion mutants, when compared to typical FL tyrosine phosphorylation by c-Abl. **(B)** Diagram illustrating the alignment of C-terminal regions of BMPR1A of various species. Boxed in red are the conserved tyrosine residues found within the region after deletion mutant #1 to carboxy end of full length receptor.

A

single Y mutants	453	457 458	467
FL	Y	Y Y	Y
A	F	Y Y	Y
B	Y	F Y	Y
C	Y	Y F	Y
D	Y	F F	Y
E	Y	Y Y	F



B

combination Y mutants			
F	F	F F	Y
G	Y	F F	F
H	F	Y Y	F
I	F	F F	F

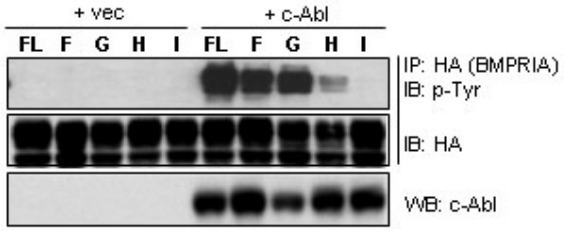


Fig. 6.3. Identifying the BMPR1A C-terminal tyrosine residues phosphorylated by c-Abl via mutagenesis.

(A) BMPR1A single tyrosine mutants do not affect the tyrosine phosphorylation status by c-Abl. Mutants A to E consist of a single (except D) mutation to replace tyrosine with phenylalanine (labeled F in blue). c-Abl was co-expressed with each of these mutants in cos7 and receptors immunoprecipitated using anti-HA antibodies. Tyrosine phosphorylation was determined by western blot. **(B)** More BMPR1A receptor mutants created from the templates of earlier single mutants. It was observed that while all 4 tyrosine residues could be phosphorylated, the major phosphorylation site for c-Abl remained at Y453/467 (mutant I consisting of all four mutated sites compared to mutant H).

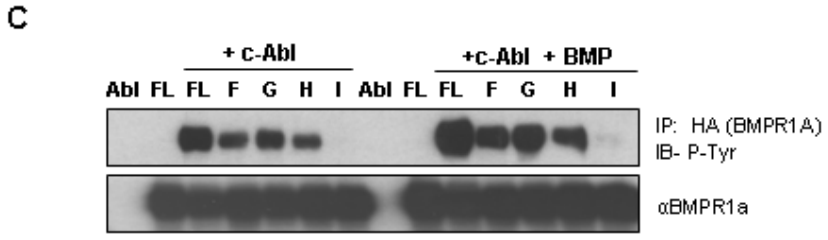


Fig. 6.3. BMP2 can enhance c-Abl mediated phosphorylation of BMPR1A on any of the all four tyrosine residues.

(C) Receptor mutants F to I, together with wild type BMPR1A [FL], were co-expressed with c-Abl in cos7 and serum starved overnight before adding 20ng/ml BMP2 for 30mins. Receptors were immunoprecipitated with anti-HA antibodies, and tyrosine phosphorylation determined by western blot. For control, lanes 1 and 2 were co-expressed with vector, and with BMP treatment, lanes 8 and 9.

6.4 BMPR1A Y453/457/458/467 mutant affects Smad1 activation, but not Smad1 binding to receptor

As BMPR1A is the bona fide activator of Smads1/5/8 and direct interaction is required, the effects of Smad1 activation was studied using a wild type Smad1 construct and as a control; a mutant Smad1 that has the last 11aa deleted so it cannot be phosphorylated by BMPR1A (structural studies showed that the C-terminal final 10 aa residues of Smad1 was shown to be flexible, while its carboxy tail end containing the final two serine residues of Ser-X-Ser, or SSXS motif was required for phosphorylation by BMPR1A (Qin *et al*., 2001, Shi and Massague, 2003). Co-expression studies revealed that Smad1 activation was compromised in the mutant tyrosine receptor, while the binding of Smad1 to the wild type or tyrosine mutant receptor remains unaffected, with the mutant smad1 as well **(Fig. 6.4).** Hence, it is likely that c-Abl enhances Smad activation via these c-terminal tyrosine residues without interfering with Smad binding to the receptor.

6.5 Mutant BMPR1A affects receptor complex formation

In what ways can c-Abl influence the receptors and affect Smad activation? Since BMPRII is needed to phosphorylate BMPRIA at the unique GS box domain in order to further activate BMPR1A itself and thus Smads, the joining of both receptor types can affect downstream events. To understand the earliest cytoplasmic steps of BMP signaling, HA and Flag-tagged constructs containing BMPR1A and BMPRII respectively were co-expressed along with c-Abl. BMPR1A was then immunoprecipitated, using HA antibody while flag antibody was applied in western

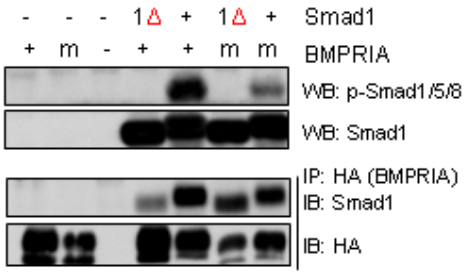


Fig. 6.4. Smad1 activation is compromised in the mutant BMPR1A.

The mutant BMPR1A [m] carrying the 4 Y-F mutations and the wild type receptor were co-expressed with Smad1, or the mutant Smad1 (Smad1Δ). Western blot analysis was used to determine Smad1 activation, using anti-phospho-Smad1/5/8 antibody, and total Smad expression using anti-Smad1 antibody. For immunoprecipitation analysis, anti-HA antibodies were used for BMPR1A wild type and mutant constructs, and Smad1 association detected with anti-Smad1 antibodies.

blot to look for the presence of BMPRII **(Fig. 6.5)**. As seen in Fig 6.5, lanes 1-3 revealed that BMPRII bound to BMPR1A, and this binding was markedly enhanced only with the addition of c-Abl, not its kinase dead form (KD). More importantly, lanes 6 and 7 show diminished binding of BMPRII, when the mutant BMPR1A was used. This signals that the 4 tyrosine sites regulated by c-Abl is needed for 'usual/normal' receptor complex formation, as the lack of these sites can reduce receptor binding to each other, and consequently a reduction in the level of Smad activation.

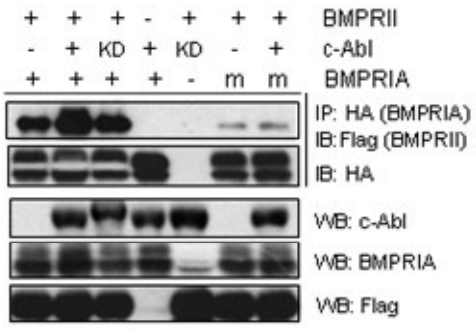


Fig. 6.5. c-Abl facilitated the interaction between BMPRII and BMPR1A.

The mutant BMPR1A [m] carrying the 4 Y-F mutations and BMPR1A wild type receptor were co-expressed with BMPRII (Flag-tagged) and c-Abl or c-Abl KD. BMPR1A was immunoprecipitated with anti-HA antibodies and the association to BMPRII were identified with an anti-Flag antibody.

6.6 Summary

Since c-Abl could augment Smad activation, it is likely that c-Abl works upstream of Smads 1/5/8 and more work was needed to understand the first tier of BMP signaling that occurs upon BMP ligand binding. Immunoprecipitation studies showed that c-Abl could tyrosine phosphorylate BMP type I receptors and endogenous tyrosine phosphorylation of BMPR1A occurred in c-Abl primary cells. In the absence of c-Abl, tyrosine phosphorylation was lower, and this was not due to differences in receptor transcript or protein expression levels (Fig 6.1). Hence, c-Abl acted on the BMP receptors and this phosphorylation action could be the main reason behind the enhanced Smad1/5/8 activation, since receptor Smads remain the only substrates of BMP receptor kinases.

In order to map the region on BMPR1A that was phosphorylated by c-Abl, serial deletion analysis of the receptor was carried out on the cytoplasmic portion of the receptor, which mainly consisted of the S/T kinase domain. It was found that all 4 truncations created could not be phosphorylated, hence the carboxy tail region of the receptor was targeted by c-Abl (Fig 6.2). Mapping of conserved tyrosine residues at this area was performed, by first aligning BMPR1A protein sequences of various mammalian species and further creating several single tyrosine mutations of the full length BMPR1A. Immunoprecipitation of these mutants showed little difference in phosphorylation status; hence more mutants were created in various combinations out of the four conserved tyrosines. Subsequently, c-Abl phosphorylation was severely abolished in mutant I containing all four tyrosines mutated to phenylalanine, with the preferred flanking tyrosine residues at Y453/Y467 favored by c-Abl (Fig 6.3).

The functionality of this receptor mutant I (Y453/457/458/467) on Smad activation was tested, and was shown that while it did not affect Smad1 binding to receptor, its co-expression with Smad1 decreased the Smad1 phosphorylation significantly, when compared to the strong Smad1 activation observed with wild type BMPR1A (Fig 6.4). More importantly, Fig 6.5 addressed the results observed in Fig 6.4, as the presence of c-Abl could enforce the binding of BMPRII to BMPR1A, while BMPRII binding to the mutant receptor was dramatically compromised, with or without c-Abl. This showed that the four tyrosine residues found on BMPR1A was essential for c-Abl to enhance receptor complex formation and thus promote receptor Smad activation, since the receptor type I and II complex was necessary for downstream Smad activation to occur.

CHAPTER 7

A NEGATIVE ROLE OF C-ABL IN BMP-INDUCED ERK1/2 ACTIVATION

With the earlier chapters demonstrating how c-Abl functions in the BMP pathway up-regulate BMP target genes via Smads1/5/8, the key mediators of another equally important BMP-induced Smad-independent pathway is studied here, in the attempt to understand the purpose for MAPK-ERK activation seen in c-*Abl*$^{-/-}$ cells (chapter 4).

The BMP-MAPK pathway is one of the pathways that were identified to be Smad-independent. However, the downstream outcomes of activating mitogenic subfamilies ERK1/2, p38MAPK and JNK, vary extensively in cell type, kinetics and magnitude (Massague, 2000). Central to BMP-MAPK signaling is the activation of TAK1 (TGF-β activated kinase), that is shown to function downstream of BMPs and TGFβ, as well as cytokines IL-1 and TNFα (Ninomiya-Tsuji *et al*., 1999). TAK1 was first identified as a MAP3K (Yamaguchi *et al*., 1995), and forms the crucial link between BMP receptors and MAPKs. TAK1, along with TAB1 (TAK1-binding protein-1), acts to mediate downstream MAPK pathways, by activating JNK and p38MAPK via MAP2Ks such as MKK3 and MKK6 (Delaney and Mlodzik, 2006, Moriguchi *et al*., 1996). TAK1 works in tandem with TAB1 as it requires TAB1 for its initial activation and autophosphorylation at Thr184/187 in its kinase activation loop (Sakurai *et al*., 2000).

TAK1 is ubiquitously expressed during early development, and generally localized in the cytoplasm (Jadrich *et al*., 2003). TAK1 serves multiple roles in mouse development, as *TAK1*$^{-/-}$ mice are embryonic lethal (Shim *et al*., 2005). An important function of TAK1 signaling lies in the regulation of cell survival. *TAK1*$^{-/-}$ MEFs are

highly sensitive to TNFα-induced apoptosis and the loss of its kinase activity attenuates the TRAIL-induced activation of NFkB-p65 and MAPKs (Choo *et al*., 2006, Thiefes *et al*., 2005). On the other hand, ectopic expression of TAK1 in induces cell death in *Xenopus* embryos (Shibuya *et al*., 1998). Here, the roles of TAK1 and TAB1 in BMP-ERK regulation are investigated.

7.1 Dual phases of ERK activation in c-Abl mutant osteoblasts is dependent on the duration of exposure to BMP2

Early on, in chapter 4, it was observed that levels of activated ERK1/2 were elevated in c-Abl mutant osteoblasts and MEFs, when compared to its wild type counterpart. c-Abl appears to have a repressive effect on MAPK/ERK activation, as in the absence of c-Abl, the level of activated ERK rises. In addition, reconstitution of viral c-Abl into knockout osteoblasts can rescue the lack of ERK inhibition in mutant cells (Fig 4.1D). To examine the pattern of this activation, and also if ERK activation can be influenced by BMP signaling, BMP2 was added at various time points ranging from 0 to 8hrs on wild type and *c-Abl*$^{-/-}$ osteoblasts **(Fig 7.1A)**. It was observed that the normal wild type osteoblasts show transient ERK activation which peaks at 30mins from BMP stimulation, and plateau at the 2hr mark, this was then followed by a consecutive repression of phospho-ERK, to levels below the basal level. It is possible that the activation and repression waves under the influence of BMP could be involved in fine-tuning $p16^{INK4a}$ expression, as ERKs1/2 are transcriptional activators of $p16^{INK4a}$ (Lin *et al*., 1998). With c-Abl mutant osteoblasts, the ERK activation pattern differs, starting with higher basal p-ERK levels, followed by extended

transient ERK activation and the lack of ERK inhibition. These differences can be better observed by means of density measurement using absolute units **(Fig 7.1B)**. Basal levels of activated MAPKK-MEK1/2, was also increased in *c-Abl*$^{-/-}$ osteoblasts, but remains constant with BMP2 induction. It is possible that c-Abl acts in an inhibitory manner on the upstream MAPKKKs, or there is a likelihood of crosstalk with the canonical RAS-RAF-MEK-ERK pathway. Nevertheless, either does not solely account for the ERK activation/repression pattern seen in mutant osteoblasts, and this underscores a novel role for c-Abl in BMP-induced ERK activity.

Besides, blocking BMP2 activity for prolonged periods with antagonist noggin and chordin reversed the repression on ERK1/2 activation in wild type osteoblasts **(Fig 7.1C)**. This supports the earlier observation that the constant presence of BMPs plays a part in ERK regulation, and likely to be partially accountable for the enhanced ERK activation in c-Abl knockout osteoblasts.

7.2 BMPR1A mutant mimics *c-Abl*$^{-/-}$ osteoblasts in ERK activation

To further understand the relationship between c-Abl, and BMP-induced ERK activation, wild type and mutant-4Y BMPR1A was overexpressed with retrovirus in C2C12, a myoblastic cell line, as well as in primary osteoblasts to observe ERK activation. In both cases expression of mutant 4Y receptor led to an up-regulation of p-ERK1/2, compared to equivalent levels of expressed wild type 1A receptor **(Fig 7.2A and B)**. This is reminiscent of c-Abl knockout osteoblasts phenotype, suggesting that the mutant receptor acts in a dominant negative fashion, and that c-Abl

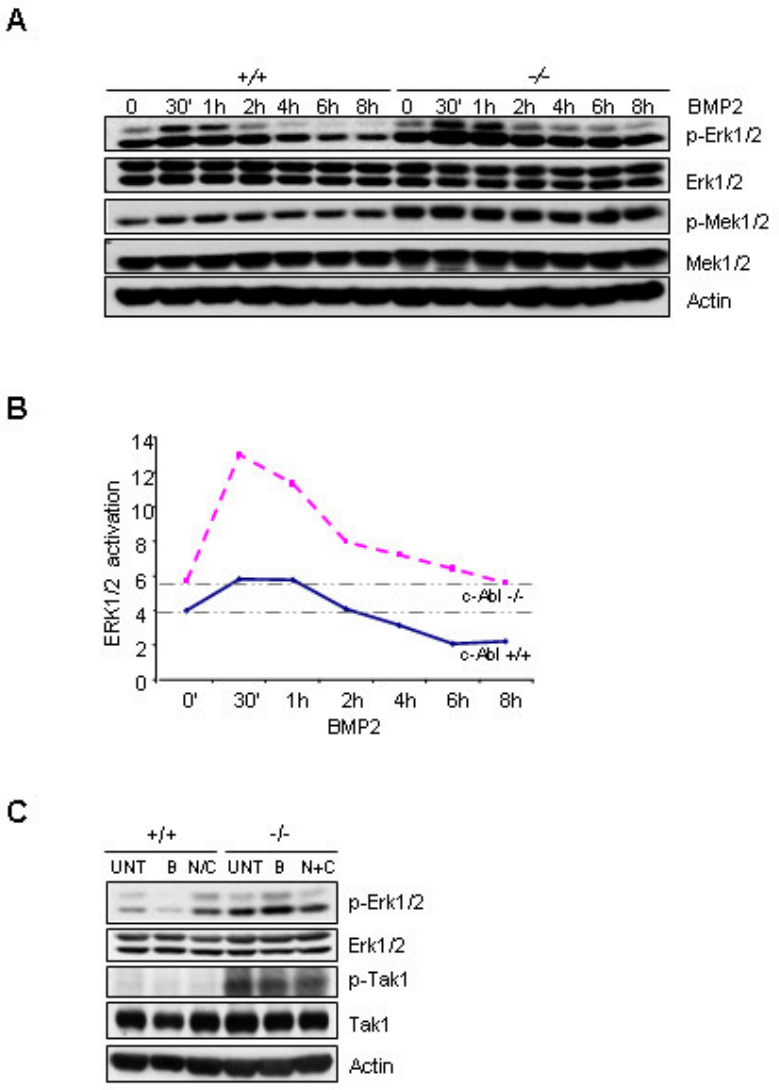


Fig. 7.1. c-Abl regulates BMP2-induced ERK activation.

(A) c-Abl deficiency altered BMP-induced ERK1/2 activation and/or suppression. 8 individual osteoblast cultures of each cell type was pooled together and subjected to various time point treatments using 100ng/ml BMP2. Western blot was then used to determine activation of various kinases. **(B)** Quantitation data of phosphorylated ERK against total ERK values. **(C)** Blocking BMP signaling led to ERK activation. Using c-Abl primary osteoblasts, BMP antagonists noggin and chordin [N+C], or 100ng/ml BMP2 [B] was added to both wild type and mutant cells for 2-3 days and cells harvested for western blot analysis.

phosphorylation of BMPR1A might be important in controlling BMP-induced ERK activation.

The TAB1-TAK1 complex has been implicated in BMP-mediated MAPK activation (Yamaguchi *et al*., 1999, von Bubnoff and Cho, 2001, Massague, 2000). It can be seen that the basal levels of activated TAK1, an upstream regulator of ERK (Hammaker *et al*., 2004), was enhanced in c-Abl mutant osteoblasts (Fig 7.1C), as well as MEFs **(Fig 7.2C)**. On the other hand, the basal p-TAK1 levels in wild type osteoblasts and MEFs remain too low to be detected, indicating that c-Abl represses the TAB1-TAK1 complex signaling. Therefore, c-Abl functions upstream at the MAPKKK, as C2C12 cells overexpressing mutant 4Y receptor also leads to higher levels of activated TAK1 (Fig 7.2A). To find out if TAK1 was indeed required for BMP-induced ERK activation, TAK1 was knocked down in C2C12 cells. Using pooled TAK1 siRNAs and a transient transfection system based from Dharmacon, cells were kept in culture for >48hrs post transfection and 100ng/ml BMP2 added directly to culture media for various time points. Compared to the mock and siRNA non-target controls, the transient ERK1/2 activation was mitigated with the addition of BMP **(Fig 7.2D)**. This implies that BMP-induced transient ERK1/2 activation utilizes TAK1, though it is unclear if c-Abl can engage TAK1 complex directly during the transient wave of ERK1/2 activation.

7.3 Role of mutant BMPR1A in TAB1-TAK complex formation

It was previously reported that the TAK1 and TAB1 molecules function downstream of BMPR1A as injection of a kinase negative form of TAK1 mRNA in *Xenopus*

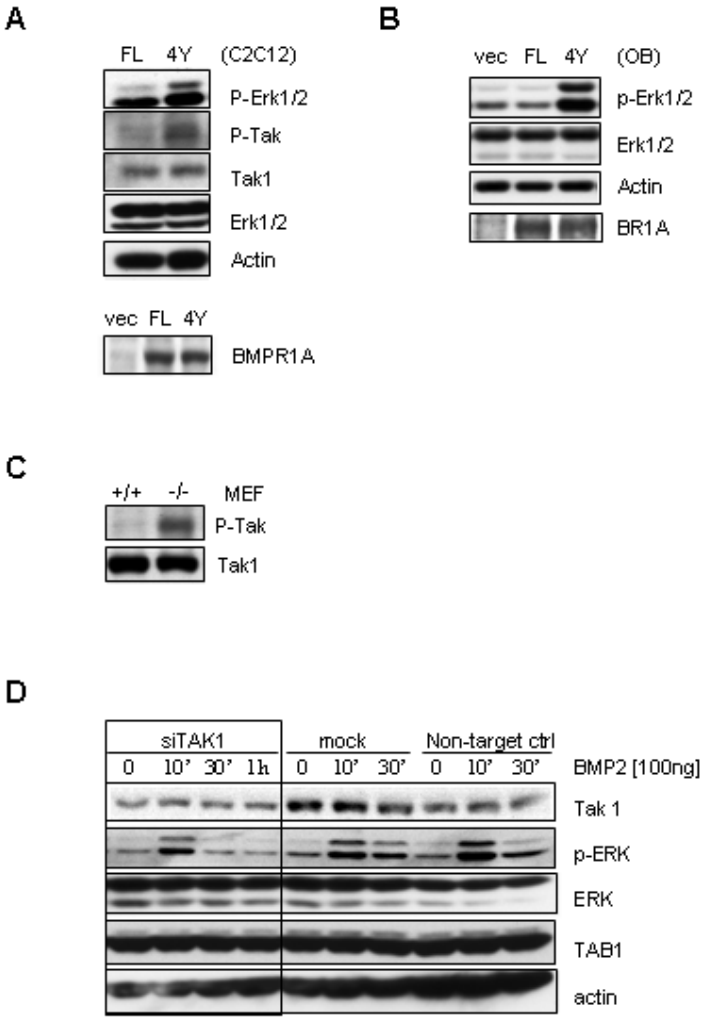


Fig. 7.2. c-Abl action on BMPR1A affects ERK activation.

Mutant BMPR1A expression leads to enhanced ERK activation in C2C12 **(A)**, as well as primary osteoblasts **(B)**. Western blots show the innate levels of p-ERK1/2 and equivalent amounts of BMPR1A in cells overexpressing wild type 1A receptor or mutant receptor, compared to vector control infected cells. **(C)** Endogenous levels of activated TAK1 protein in c-Abl wild type and mutant MEFs. **(D)** Tak1 is required for transient BMP-induced ERK activation. TAK1 is silenced in C2C12 cells for 2 days and BMP2 added for short time points. Cells were harvested for western blot and probed for total TAK1 and p-ERK1/2 expression.

embryos could revert the ventralization phenotype generated by constitutive BMPR1A activation that reproduced the effect of BMP2/4 (Shibuya *et al*., 1998). Essentially, it is important to look into the physical interaction of these molecules at the BMP receptor level as they are needed to tranduce the downstream effect of observed ERK activation seen in *c-Abl*$^{-/-}$ osteoblasts. Co-immunoprecipitation experiments in cos7 cells using mutant-4Y receptor showed that TAB1 displayed a higher affinity for the mutant BMPR1A compared to wild type receptor **(Fig 7.3A)**. This experiment was repeated three times, with the average difference of 2.2 fold increase with density quantitation. This enhanced interaction could be direct, or mediated by endogenous proteins such as XIAP. Importantly, these results suggest that c-Abl deficiency can facilitate recruitment of TAB1-TAK1 complex to BMPR1A, and hence augment ERK1/2 activation. **Fig 7.3B** summarizes the results of all chapters, illustrating BMP activation of a Smad-dependent and Smad-independent pathway that converge to fine-tune the expression of $p16^{INK4a}$ which mediate c-Abl's effect on osteoblast expansion.

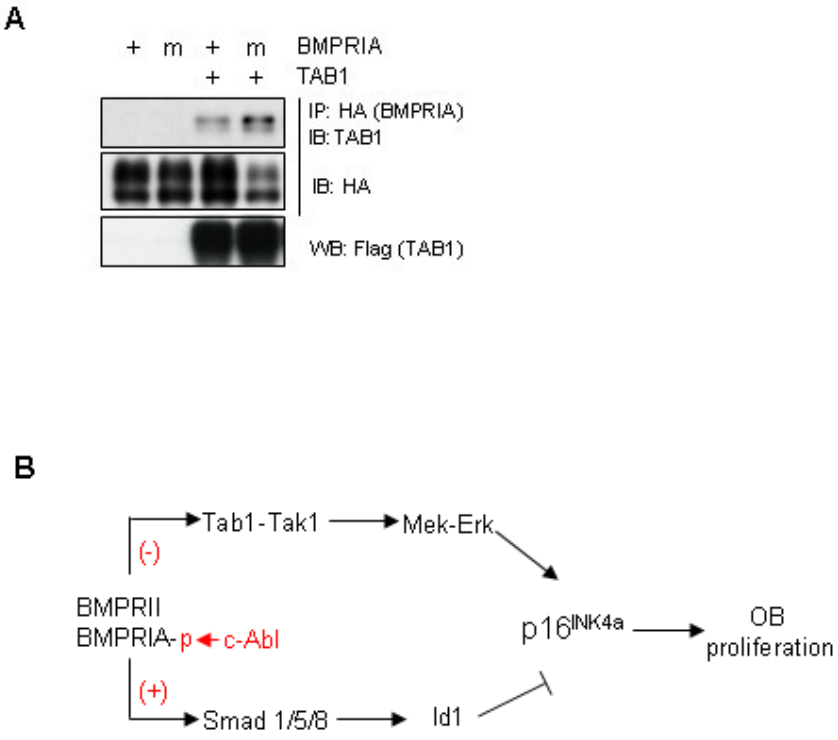


Fig. 7.3. c-Abl phosphorylation of BMPR1A counters TAB1 interaction.

(A) TAB1 was shown to display higher affinity for mutant 1A receptor [m] than wild type counterpart. Flag-tagged TAB1 and HA-tagged 1A receptors were co-expressed in cos7 cells. Receptors were immunoprecipitated using anti-HA antibodies and co-purified TAB1 detected by western blot. **(B)** A diagram showing how c-Abl might be involved in regulating osteoblast expansion in response to BMPs.

7.4 Summary

BMP signaling in osteoblasts gave rise to divergent outcomes of the MAPK-ERK1/2 regulation. A short duration of BMP2 exposure (about 30mins) resulted in ERK activation, while long term BMP stimulation caused ERK repression. This suggests existence of a negative feedback regulation on BMP-induced ERKs, which was further confirmed by enhanced phospho-ERK levels when BMP signaling was impeded by adding BMP antagonists in the culture media (Fig 7.1C, lane 3). Yet, careful analysis of ERK activation by comparing *c-Abl*$^{-/-}$ with wild type cells revealed that the transient activation phase was not affected by c-Abl deficiency. Instead, differences could be observed with ERK repression at the longer time range of BMP2 stimulation. *c-Abl*$^{-/-}$ osteoblasts originated with elevated basal phospho-ERK levels, comparable to the levels seen in noggin/chordin treated cells, yet repression of phospho-ERK was either delayed (Fig 7.1B), or lost (Fig 7.1C, lane 5), with long periods of BMP treatment. Moreover, cells expressing the mutant-4Y receptor also displayed higher phospho-ERK amounts in comparison to wild type BMPR1A (Fig 7.2A and B), suggesting that tyrosine phosphorylation of BMPR1A by c-Abl serves some function in BMP-induced ERK repression.

On the other hand, the mechanism by which BMPs regulate MAPK-ERK is not well understood. Previous studies suggest that BMPs might activate MAPKs through TAB1-TAK1 complex, joined by adaptor protein XIAP (Yamaguchi *et al*., 1999). XIAP was found to recruit this complex to BMPR1A, and this could subsequently result in MAP3K-TAK1 activation. Indeed, TAK1 plays a positive role in BMP-induced ERK activation, as knockdown of Tak1 resulted in lower phospho-Erks at the basal level in response to BMP2 induction (Fig 7.2D). Moreover, TAB1 was found to

bind better to the mutant BMPR1A (Fig 7.3A), and since TAB1 is needed to activate TAK1, this correlates well with the subsequent observed elevated downstream activation of MAP3K-TAK1, MAPKK-MEKK1 and finally, MAPK-ERKs, in c-Abl deficient cells, as well as cells overexpressing mutant BMPR1A.

CHAPTER 8

BMP SIGNALING IN BCR-ABL AND THE SUSTAINANCE OF BONE MARROW PROJENITOR CELL POPULATION

Studies described in the previous chapters support the notion that the cytoplasmic form of c-Abl acts on BMP type 1 receptors to affect downstream signaling to enhance Id1 expression, to down-regulate Erk1/2 activation, leading to repression of $p16^{INK4a}$ expression. As a result, the net outcome favors osteoblast proliferation. Another perspective into Abl signaling would be to understand the etiology of BCR-ABL oncogenesis in the development of CML.

BCR-ABL expression is required for the maintenance of CML. What are the signaling pathways involved? BCR-ABL targets many common signaling pathways to block apoptosis, promote proliferation and affect cell adhesion characteristics, processes that are advantageous for cellular transformation. The PI3K-AKT pathway is activated by BCR-ABL, likely to target AKT-mediated inactivation of FoxO transcription factors that affect apoptosis through p27Kip1 (Gesbert *et al*., 2000) and Bim (Kuribara *et al*., 2004). Proliferation is promoted by activation of the mitogenic Ras-Raf-MEK-ERK pathway, by recruitment of adaptors Grb2-Gab2 (Million and Van Etten, 2000). Yet, specific roles for BCR-ABL activation of p38-MAPK and repression of ERK was reported (Kohmura *et al*., 2004). The JAK/STAT pathway is involved in BCR-ABL transformation as STAT5a could abrogate hematopoietic cell dependence on cytokines (reviewed in Steelman *et al*., 2004). BCR-ABL can also alter actin cytoskeleton dynamics by binding to cytoskeletal proteins such as Sos-1

(Sini *et al*., 2004), and activating related pathways to enhance cell adhesion to the extra-cellular matrix (Feller, 2001).

From the current understanding of BCR-ABL in signaling pathways, this chapter looks into the existence of a senescence pathway that is repressed in BCR-ABL positive cells.

8.1 BCR-Abl potentiates BMP-induced Id1 expression, and can be inhibited by imatinib/STI571 treatment

Using BCR-ABL positive CML human cell line K562, the endogenous protein levels of Id1 and phosphorylated smads1/5/8 were found to be highly expressed, when compared to its negative counterpart (myeloblastic cell line HL-60) **(Fig. 8.1A)**. This suggests that increased Smad-Id1 signaling correlate with higher levels of Abl kinase activity.

A preliminary look at downstream effects of suppressing BCR-ABL activity by adding inhibitor imatinib into culture media for 1 and 3 days demonstrated that basal Id1 expression is completely abolished even in K562 cells in 1 day, or 3 day cultures **(Fig. 8.1B)**.

In addition, the presence of BMP2 in culture could further induce Id1 expression after 6 hrs of treatment **(Fig. 8.1C, lanes 5 and 6)**, with the response more pronounced at the transcript levels when compared to HL60 cells **(Fig. 8.1D)**. Interestingly, pretreatment of cells with imatinib prior to BMP2 treatment led to a huge reduction of

Id1 induction under BMP stimulation (**Fig. 8.1C-D, lanes 4 and 8**). This shows that BCR-ABL positive cells respond to BMPs, and it is due to this active BMP signaling that Id1 is enhanced. It appears that Id1 serves as an important target of BCR-ABL kinase activity, which is effectively repressed upon imatinib treatment.

Likewise, limiting the canonical BMP2/4 circulation in culture media using BMP antagonists noggin and chordin at recommended ED_{50} dosage showed that the high levels of basal Id1 in K562 cells could be reduced by about more than half after 72 hrs **(Fig. 8.1E)** This implies that a positive feedback mechanism of BMP signaling pathway exists in myeloid cell types , and is in part needed to maintain constant, high levels of Id1 expression, along with other pathways. The similar fashion in which HL60 cells behave in response to noggin and chordin confirms that Abl in essence, rely on Id1 as a positive downstream mediator in encouraging survival or growth, albeit with the "amplified" Id1 level in BCR-ABL cells, it may possibly promote aggressive growth signals.

8.2 $p16^{INK4a}$ expression and the ageing of the bone marrow cells

In vivo, osteoblasts reside in the bone marrow, and to gain insight into the influence of its microenvironment, bone marrow cells were pooled from adult mice of three age groups; at 4, 12 and 17 months to look at the effects of age on HSCs that consist of virtually all progenitor precursor cell populations of myeloid lymphoid and erythroid lineage. $p16^{INK4a}$ expression at the protein level in these bone marrow cells increased with age **(Fig. 8.2A)**, while this can be more clearly seen at the mRNA expression **(Fig. 8.2B)**. The concomitant increase in $p16^{INK4a}$ expression with increasing age have

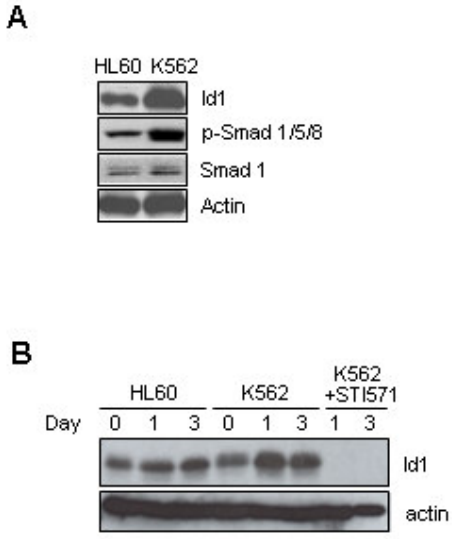


Fig. 8.1. Profile of Id1 gene regulation in an innate activated Abl cell line model. **(A)** Constitutive BCR-ABL activity in K562 cells showed that endogenous Id1 protein expression was elevated, along with activated BMP-Smads 1/5/8 when compared to BCR-ABL negative HL60 cells. **(B)** HL60 and K562 suspension cells were counted and seeded in T25cm^2 flasks, at a cell density that encouraged proliferation (with exception of day 3 - K562 cells seeded at a lower density to parallel growth rate for HL60 cells as K562 cells proliferate faster). Cells were then harvested for western the following day (day 0), followed by days 1 and 3. In lanes 7 and 8, 1uM imatinib was added to culture media from day 0.

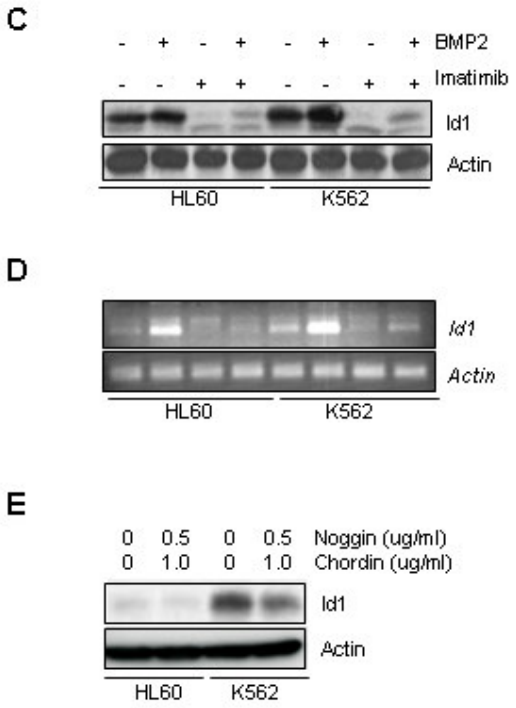


Fig. 8.1. BCR-ABL can potentiate Id1 expression via the BMP pathway.

(C) Western blot shows that imatinib mesylate can overcome BMP-induced Id1 in K562 cells. Cells were pretreated with 1uM imatinib for 4 hrs, before adding 100ng/ml BMP2 for 6hrs. **(D)** RT-PCR shows mRNA profile of Id1 under the same conditions used in Fig 8.1C. **(E)** Blocking BMP signaling by adding noggin and chordin for 3 days down-regulates Id1 protein expression in both K562, as well as HL60 cells.

been reported in normal human and mice tissues, and may reflect a role for $p16^{INK4a}$ in cellular senescence *in vivo* (Nielsen *et al*., 1999). Normal $p16^{INK4a}$ -mediated aging may take place in the bone marrow, with increasing intensity at an older age, as senescence of bone marrow stroma may decrease the ability of marrow cells to form osteoblast precursors (Manolagas and Jilka, 1995)

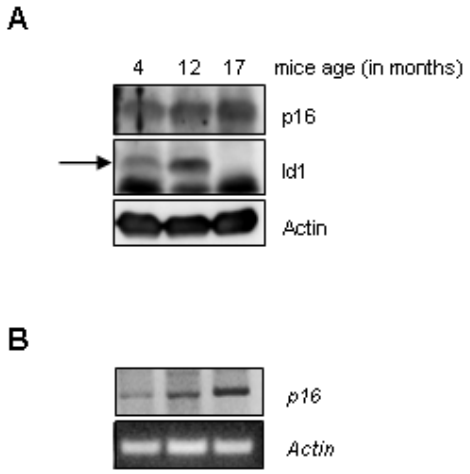


Fig. 8.2. p16^{INK4a} expression in the bone marrow of aging mice.

(A) Bone marrow cells were extracted from long bones (femur and tibia) of mice. 3 mice of each age group; 4mths, 12 mths and 17mths were pooled. Cells were rid of red blood cells, and lysed with protein lysis buffer for western blot analysis to probe for p16^{INK4a} and Id1 expression. **(B)** Bone marrow cells were obtained from the above mentioned and cell pellet subjected to RNA extraction, RT-PCR, to measure p16^{INK4a} transcripts.

8.3 Summary

From the above results, it can be concluded that a BMP-Id1 pathway also exists in BCR-ABL positive cells, and mirrors the positive role that c-Abl plays in bone development. Id1, responsible for promoting proliferation signals and inhibiting initiation of differentiation programs, is heavily amplified in BCR-ABL positive cells. With K562 cells, the pattern of increased Id1 induction reflects the intensity, or the degree of innate Abl kinase activity, which was effectively suppressed upon imatinib treatment (Fig 8.1A and B). This suggests that the sustained, yet stable expression of high levels of Id1 is highly dependent on BCR-ABL kinase activity, along with other tyrosine kinases that imatinib/STI571 inhibits.

This phenomenon was enhanced with the addition of BMP2, with Id1 up- regulated at the mRNA level. Imatinib also severely repressed BMP-induced Id1 (Fig 8.1C and D). The BMP-Smad pathway forms an integral part of the many pathways BCR-ABL targets (Steelman *et al*., 2004), since the lack/inhibition of BMP signaling feedback (Fig8.1E) and adding imatinib to osteoblasts give a similar reactions; both represses Id1 expression, along with down-regulation of activated Smads (results not shown), in contrary to the presence of an intact, overactive BMP system in K562 cells.

Also, a salient feature of chronic phase CML is the subtle but consistent increase in immature and mature myeloid cells, which later develops into abnormally high levels of immature myeloblasts (blasts crisis phase) (Wong and Witte, 2004). Blasts cells originate from the bone marrow. Cellular barriers to such aggressive tumorigenesis must be in place, and $p16^{INK4a}$ –mediated stem cell senescence might be one such barrier. The analysis of bone marrow stomal cells in wild type mice of various ages

also demonstrates the concomitant increase of $p16^{INK4a}$ (Fig 8.2). While Id1 expression diminish in 17-18mth old mice, surmises the fact that regulation of $p16^{INK4a}$ expression may be largely controlled by Id1, and the role of BMPs in the marrow microenvironment cannot be discounted.

BCR-ABL induced Id1 via BMP-Smads in CML is complicated by the multitude of other intracellular signaling pathways taking place. Nevertheless, the main reasons could be to target and block $p16^{INK4a}$ -mediated senescence, while promoting aberrant clonal cell expansion. Indeed, it was reported that BMPs play a role in mouse ES cells was to encourage self-renewal, rather than block ES cell differentiation in neural crest formation (Varga and Wrana, 2005). Yet, it is also possible that BMPs have more discrete roles on ES cells in post-natal physiology (or adult tissue regeneration), and the emphasis might shift to regulate cell expansion and survival.

Hence, these results provide an insight into how CML disease state is maintained in the cells, and the implications of these findings are further discussed in chapter 9.

CHAPTER 9

DISCUSSION AND FUTURE PERSPECTIVES

A link for osteoblast premature senescence and aging associated osteoporosis

c-Abl knockout mice display aging associated osteoporosis, characterized by defects in osteoblast function and numbers, but not osteoclasts (Li *et al*., 2000). *Ex vivo* analysis of *c-Abl*$^{-/-}$ osteoblast cultures painted a profile that matched this phenotype, as isolated mutant osteoblasts have reduced proliferation capacity, along with accelerated senescence as demonstrated by increased β-galactosidase staining and $p16^{INK4a}$ expression. This premature senescence could be mediated by increased $p16^{INK4a}$ expression, resulted from increased ERK1/2 activation and decreased Id1 expression (Chapter 4). In adults, constant bone remodeling is essential for bone homeostasis and repair, which accounts for an estimated replacement of 25% of trabecular bone and 3% of cortical bone annually (Manolagas and Jilka, 1995). A reduction in the growth potential of osteoprogenitors can lead to undesirable consequences, such as an insufficient supply of mature osteoblasts, or the induction of senescence which might also interfere with the function of neighboring cells (Chan and Duque, 2002). In addition, *in vivo* studies on the long bones of *c-Abl*$^{-/-}$ adult mice revealed higher levels of $p16^{INK4a}$ staining in marrow region near the hyperchondrial growth plate, and higher levels of $p16^{INK4a}$ mRNA, while lesser number of osteoblasts per bone surface was reported. This provides a rationale for *c-Abl*$^{-/-}$ mice as a suitable mouse model to associate premature osteoblast senescence with senile osteoporosis. The concept that cellular senescence contributes to tissue aging, such as osteopenia of bones, parallels the study of PASG, a proliferation associated SNF2-like gene in

which its deficiency led to premature aging phenotypes, including osteoporosis (Sun *et al*., 2004).

Another feature associated with aging is cell susceptibility to stress. Cells of patients with progeroid syndromes were more prone to hypersensitivity to stress-induced cell death. c-Abl have been implicated in cellular response to oxidative stress, and it was found that *c-Abl*$^{-/-}$ osteoblasts and *c-Abl*$^{-/-}$ *Argl*$^{-/-}$ MEFs showed higher susceptibility to oxidative damage (Li *et al*., 2004, Cao *et al*., 2003). Oxidative stress have also been known to induce senescence in many cell types, and it is important to determine if the up-regulation of p16^{INK4a}, and decrease in Id1 seen in senescent *c-Abl*$^{-/-}$ osteoblasts were the consequence of a response to oxidative stress, apart from premature senescence. Experiments using hydrogen peroxide to induce oxidative damage on c-Abl osteoblasts, and conversely the use of ROS (reactive oxygen species) blocker; NAC (N' acetylcysteine), did not alter the expression of p16^{INK4a} and Id1 significantly, although senescence was induced in the former (results not shown). This confirmed that changes in p16^{INK4a} or Id1 expression remains unaffected by oxidative stress-induced senescence, hence the hypersensitivity to oxidative stress and premature senescence in *c-Abl*$^{-/-}$ osteoblasts are independently modulated by c-Abl.

This study highlights the importance of p16^{INK4a} in the aging process of osteblasts. Premature senescence of *c-Abl*$^{-/-}$ osteoblasts were found to be the result of p16^{INK4a} up regulation, consistent with the studies by other groups that report the accumulation of p16^{INK4a} in aged cells and the reduction of Id1, or cells deficient for p16^{INK4a} are resistant to senescence (Huot *et al*., 2002, Alani *et al*., 2001). Moreover, the interest in p16^{INK4a} mediated aging was renewed when up regulation of p16^{INK4a} was shown to

induce an aging-dependent decrease of forebrain progenitors and pancreatic beta-cells (Molofsky *et al*., 2006, Krishnamurthy *et al*., 2006).

Nonetheless, *c-Abl*$^{-/-}$ mice displayed other phenotypes reminiscent of an aging organism such as premature death, thymus atrophy and lymphopenia, other than osteoporosis. Taken together, the mouse phenotypes, the hypersensitivity to oxidative stress, and the premature senescence of osteoblasts suggest that c-Abl plays a role in bone remodeling and general organismal aging. The role of p16^{INK4a} up-regulation and BMP activated pathways may also contribute to c-Abl function in other aspects of development and homeostasis.

A role for c-Abl in BMP signaling, ERK1/2 activation and Id1 induction

In chapters 4 and 5, ERKs, and Id1 were identified as major players involved in p16^{INK4a} mediated aging of *c-Abl*$^{-/-}$ osteoblasts. Without c-Abl, excessive MAPK-ERK activation and Id1 repression was observed. This was in line with established studies that stated ERKs to be positive regulators, while Id1 a negative regulator of p16^{INK4a}, and Id1 along with p16^{INK4a}, influenced the senescence of human and murine cells (Lowe and Sherr, 2003).

BMPs are secreted by osteoblasts and are required for bone formation and regeneration (Chen *et al*., 2004, Tsuji *et al*., 2006). It was observed that blocking BMPs with antagonists noggin and chordin could up regulate p16^{INK4a}, while long term BMP2 treatment down-regulated p16^{INK4a} levels. These findings suggest that BMPs modulate p16^{INK4a} expression via the ERK1/2 and Smad1/5/8-Id1 pathways. Yet, p16^{INK4a} regulation may be more complicated due to inputs of ERK1/2 from

growth factors, as well as the feedback regulators of BMP-Smad signaling such as inhibitory Smads. However, the Id1 and ERK signaling pathways that govern $p16^{INK4a}$ expression were found to act independent of each other as Id1 remains unaffected by MEK1/2 inhibition with U012S (MEK1/2 activates ERK1/2 -Fig 4.1B). The fact that c-Abl affects ERK activation and Id1 hints at the possibility of a BMP-Smad and BMP-Smad-independent pathway taking place, as c-Abl have been shown to participate in TGFβ activated, Smad-independent pathway (Daniels *et al.*, 2004, Wang *et al.*, 2005). Thus it remains likely that the BMP pathway operates in cooperation with other signaling pathways to control $p16^{INK4a}$ transcription, as in the case of stem cell regulation (Zhang and Li, 2005).

Furthermore, these findings suggest that BMPs play a role in osteoprogenitor expansion. We found that Id1, when overexpressed could extend the lifespan of *c-Abl*$^{-/-}$ osteoblasts, indicating the down-regulation of Id1 by c-Abl deficiency contributed to the reduced proliferation capacity of *c-Abl*$^{-/-}$ osteoblasts, via $p16^{INK4a}$. Since BMP inhibition by noggin and chordin not only impedes osteoblast differentiation, but also cause the reduction of Id1 and elevation of $p16^{INK4a}$ in wild type cells, implies that autocrine BMPs may function to promote osteoblast proliferation via Id1. This idea is in agreement with the finding that targeted noggin overexpression in osteoblasts led to the depletion of osteoprogenitor cells of 8 month old mice (Wu *et al.*, 2003). The question remains on how BMPs work in unison with other pathways to regulate osteoblast expansion and osteoblast differentiation.

c-Abl modulates BMP receptors and negates BMP-Tak1 signaling

c-Abl was able to up-regulate Smad activation by modifying BMP type I receptors, whereby the tyrosine residues found at the C-terminus was necessary for robust complex formation of type I and type II receptors. It was speculated that type IV c-Abl, which harbors a myristoylation signal and is potentially attached to the plasma membrane, was responsible for this phosphorylation. On the other hand, the expression pattern of BMPR1B was limited to various embryonic tissues; while BMPR1A could be ubiquitously expressed in developing embryos (Kawabata *et al*., 1998) provided a rationale to study BMPR1A regulation by c-Abl. Nevertheless, a similar mode of action on BMPR1B by c-Abl would be probable since both BMP type 1 receptors, BMPR1A and BMPR1B; possesses 85% aa sequence identity similarity to each other and they share almost the same conserved tyrosine residues. The functions of both type I receptors appear to confer unique roles in bone development (Kawakami *et al*., 1996, *in vitro* studies by Chen *et al*., 1998 and Kaps *et al*., 2004), yet overlapping functions were also reported (Yoon *et al*., 2005). Nevertheless, it appears that BMPR1A is ultimately needed for induction of mesoderm formation /embryogenesis since *bmpr1a*$^{-/-}$ mice are embryonic lethal (Mishina *et al*., 1995), compared to the less severe phenotype of null mutation in BMPR1B gene. Comparatively, the phenotypes of *c-Abl*$^{-/-}$ mice could provide clues to understand the functions of BMP receptors in neo-natal and post-natal development, such as and the maintenance of tissue homeostasis and repair, this importance is stressed in mouse postnatal bone formation by Zhao *et al*., 2002.

It also appears that BMPRII, harboring a large cytoplasmic tail of about 600 aa following the kinase domain, does not play a role in c-Abl regulation of the BMP signaling, but is required for BMPR1A activation. Indeed it was previously reported

that this tail domain held little significance for BMP-Smad mediated signaling, though it may play a role in mediating other pathways (Wieser *et al*., 1993, Nishihara *et al.*, 2002). In our study, a novel role for the C-terminal domain of BMPR1A is uncovered, other than its function in Smad activation.

The findings that c-Abl regulates BMPR1A adds to the list of cited examples of BMP receptor post-translational modification by kinases. BMPRII was found to be regulated by interaction with LIM kinase (Foletta *et al*., 2003), c-kit, a stem cell factor receptor (Hassel *et al*., 2006) c-Src; a c-Abl related kinase (Wong *et al*., 2005), and TrkC (Jin *et al*.,2007) while GD5-mediated BMPR1B signaling was affected by receptor tyrosine kinase Ror2 (Sammar *et al*., 2004). However, the functional significance of some of these kinase-receptor interactions has yet to be validated in *in vivo* settings. Apart from phosphorylation, other modes of modification have also been reported. Sulfation was found to be required for BMP2 induction of Id1 (Osses *et al*., 2006), while pamitylolation was found to occur on BMP receptors (by personal correspondence with Dr. Leong Wai Fook), and epigenetic silencing of BMPR1B determines cell-fate decision of glioma stem cells (Lee *et al*., 2008). The ever increasing discovery of various post-translational events on BMP receptors shows the complex regulation of an apparently simple three-tier signaling pathway.

Also, steps taken to study the structural and/or physical aspects of receptor regulation provide better understanding of receptor dynamics in the cell membrane. Pre-complexed BMPR1A and BMPRII at the plasma membrane (or PRCs- Pre-formed receptor complexes) forms a precedence for canonical receptor-Smad activation, while in the case of latent complex formation induced upon BMP ligand binding (or

BISCs- BMP-induced signaling complexes) leads to non-Smad MAPK signaling (Nohe *et al*., 2002) Hence, this supports the data that c-Abl facilitation of pre-complexed IA and II, enhances Smad activation and thus transcription of downstream target genes such as Id1. In addition, BMP receptors exist in a dynamic state as they not only form clusters or enact rearrangement upon BMP induction, but also interact with caveolae compartments (Nohe *et al*., 2003 and 2004), and exhibit different modes of BMP receptor endocytosis that pre-determines preference for Smad-dependent or Smad-independent signaling (Hartung *et al*., 2006). c-Abl might also regulate the BMP receptors via other mechanisms such as the ones mentioned above, or to affect receptor degradation, and is worth exploring further in the future.

The consequent activation of BMP receptors at the cell membrane upon BMP ligand binding not only triggers the receptor Smad activation cascade, but also the lesser known Tak1-MAPK pathway. In chapter 7, the role and importance of TAK1 in BMP-MAPK signaling is explored. ERK1/2 was found to be repressed with long durations of BMP treatment, while a transient activation is initially observed within 30 mins of BMP addition. What is the role of ERK activation and repression in the time frame of BMP signaling? We argue that the function for ERK activation and repression by c-Abl might serve disparate purposes, and although ERK activation involves Tak1 and its activation is heightened in *c-Abl*$^{-/-}$ osteblasts, this could reflect the consititutve general repressive action of c-Abl on ERKs. However, c-Abl activation of ERKs, albeit a short one, may be important in regulating or work in synergy with other growth related MAPK pathways to promote proliferation. To a lesser extent, another possibility would be to hinder BMP-Smad signaling as ERKs can phosphorylate the linker region of receptor Smads and prevent its translocation

(Massague, 2003). Hence, the transient activation of ERK under BMP, might also contribute to the phenotype seen in *c-Abl*$^{-/-}$ mice through activation of other pathways.

On the other hand, what do the repression of ERK1/2 in BMP signaling serves in the context of bone development? An example of BMP signaling required for ERK repression was to counter key effectors (ERK being one of them) of the FGF pathway in the growth plate region of bone (Yoon *et al*., 2006). ERK repression was also found to be required for BMP induction of early osteoblast genes in human marrow cells (Osyczka and Leboy, 2005). Long term BMP-induced inhibition of MAPK/ERK may also have an influence on cell survival, as TGFβ family members have been reported to enlist MAPKs in activation of apoptotic pathways (Massague, 2000). The XIAP adapter protein, constitutively bound to BMPR1A and interacts with Tab1 (Yamaguchi *et al*., 1999), is likely to be involved, and could also be the link for increased apoptotic osteoblasts seen in the TUNEL immunostaining of *c-Abl*$^{-/-}$ long bones (Fig 3.3), as it has been reported that BMP signaling is necessary for induction of apoptosis of neural progenitors, by the combined actions of Smad and TAK1 (Kendall *et al*., 2005). It will be interesting to further study the role of XIAP, as well as if the apoptotic signaling cascade is triggered, as BMP-MAPK signaling is favored due to defective BMP-Smad pathway in *c-Abl*$^{-/-}$ mice.

In our study, c-Abl acts to stabilize BMP receptor complex formation, which augments Smad activation. Concurrently, this c-Abl-BMPR1-BMPRII complex acts to repress the action of TAK1-TAB1 activation by physical interference. The subsequent increase in Id1 and down-regulation of active ERKs works hand in hand to fine-tune $p16^{INK4a}$ gene expression, so it can be inferred that BMP-TAK1 activation

serves to oppose canonical BMP-Smad pathway in *c-Abl*$^{-/-}$ osteoblasts, summed up earlier in Fig 7.3B. Nevertheless, more work will also be needed to understand the mechanism of BMP-MAPK signaling and its influence on BMP-Smad signaling, emphasized in Qi *et al*., 2004, and Zhang and Li, 2005. This is because the negative impact of TAK1 activation on BMP-Smad activation could be far greater than anticipated as activated TAK1 has been shown to bind to all Smads and Tak1 overexpression attenuated the BMP-dependent differentiation potential, as well as block the transcription of osteogenic marker genes in C3H10T½ cells (Hoffman *et al*., 2005). Therefore, the subtle actions of c-Abl on BMP receptors exemplify the multitude stages of BMP regulation that affects the transcriptional outcome and thus the cell's response to BMPs.

BMPs and Abl kinases; involvement in oncogene-induced senescence

In retrospect, the previous findings raised some important questions on Abl function. The phenomenon of replicative senescence is postulated to act as an important barrier against neoplasticity and tumor formation (Sherr and DePinho, 2000, Mathon and Lloyd, 2001), and can be seen in normal cells harboring enforced expression of cancer-promoting genes such as Ras; termed oncogene-induced senescence, which requires both intact p53 and $p16^{INK4a}$-Rb pathways (Braig *et al*., 2005, Benanti and Galloway, 2004). While it was shown that the loss of c-Abl induces high $p16^{INK4a}$ expression and leads to premature osteoblast senescence, BCR-ABL on the other hand, was also able to activate BMP-Smad-Id1 pathway and it is likely that BCR-ABL prevention of regular stem cell senescence acts as a viable mechanism to support HSC transformation.

There is other evidence to support this notion. Firstly, the persistent and sustained high levels of Id1 expression seen in BCR-ABL positive cells was likely to act as an indispensable target that drives indefinite self-renewal capacity and expansion of myeloid progenitors, possibly at different time frames of cell fate decision. Though the role of Id proteins demonstrated some functional redundancy in the immune system, specificity of each Id protein function was essential. It was found that Id1, and not Id3, maintains long-term repopulating HSC cell expansion (Perry *et al*., 2007), while Id2 expression peaked in cells undergoing terminal myeloid differentiation (Ishiguro *et al*., 1996). Also, the ectopic expression of BCR-ABL could sustain the self-renewal of mouse ES cells (Nakamura *et al*., 2005).

It appears that Id1 up regulation by BCR-ABL serves as a signaling midpoint in the cross-talk and activation of many pathways. Id1 was singled out in several examples in the study of BCR-ABL malignancy such as; to promote invasiveness of leukemic cells via matrix metalloproteinase 9 (MMP9) (Nieborowska-Skorska *et al*., 2006), via Akt inactivation of FOXO3a in K562 cells (Birkenkamp *et al*., 2007) and C/EBPα mediated myeloid differentiation (Wagner *et al*., 2006). Id1 also participate in promoting angiogenesis (Sikder *et al*., 2003, Ruzinova *et al*., 2003), while *c-Abl*$^{-/-}$ mice showed defects in angiogenesis (Nunes *et al*., 2001) which could in part be due to compromised Id1 levels.

Secondly, another essential function of Id1 is to regulate cell cycle events, via suppression of $p16^{INK4a}$, along with other cyclin-dependent kinases and/or inhibitors. Despite the multitude of proteins that keep $p16^{INK4a}$ expression tightly controlled, Fig 8.2 demonstrated that $p16^{INK4a}$ expression stably increases with age, while Id1

expression is lost, in the bone marrow of aged heterologous mice. Therefore, it appears that a 'theshold' level of Id1 expression is required in the bone marrow to oppose $p16^{INK4a}$–mediated senescence, though it will be of interest to find out if the individual cell types found in the bone marrow exhibit similar profiles with age. Nevertheless, Morrison and team studied the *in vivo* properties of murine marrow cells and found that old mice had functionally incapable HSCs compared to young mice, despite being proliferative (Morrison *et al*., 1996). It is not fully understood if replicative senescence can only be triggered following cessation of cell expansion or that they can both occur concurrently, but Id1 plays important roles in these two outcomes of cell fate, and its time and amount of expression may be the determinant. This also raised the question of whether intrinsic determinants or the aging microenvironment could give rise to leukemia in aging subjects. Since $p16^{INK4a}$ has been implicated on the phenomena of stem cell aging (Janzen *et al*., 2006), it is imperative to embrace the role BMPs and senescent osteoblasts play in the bone marrow microenvironment as well. BMP signaling have been indirectly implicated in study that cited the lack of JunB gave rise to a CML like disorder in mice, stemming from HSCs (Passegue *et al*., 2004), since JunB is a BMP target gene. c-Myc, another transcription factor that also can be induced by BMP2, triggers $p16^{INK4a}$ mediated senescence in tumor cells when inactivated (Wu *et al*., 2007). More importantly, the role of osteoblasts is to provide a stem cell niche for HSC expansion in the marrow, and failure to maintain this niche may underlie the pathogenesis of senile osteoporosis (Calvi *et al*., 2003, Zhang *et al*., 2003, Visnjic *et al*., 2004 and reviewed by Calvi, 2006). Our findings suggest that BCR-ABL enhanced expression of Id1 and repression of $p16^{INK4a}$ might be mediated by the BMPs circulating in the bone marrow, while BMPs are secreted by osteoblasts. Hence the secondary effect of aging

osteoblasts and disrupted BMP signaling might overall contribute to other immune cell defects as well as development of leukemias.

Moreover, it was found that the expression of $p16^{INK4a}$ was silenced in K562 cells (data not shown), as well as in leukemic cells of many CML patients, indicating the importance of down-regulating $p16^{INK4a}$ in CML development. This was exemplified with an example of $p16^{INK4a}$ repression by *Bmi-1* that led to premature senescence of stem cells (Itahana *et al*., 2003).Hence, expressing high levels of Id1 contributes to delaying and/or inhibiting the onset of senescence program of myeloid cells by keeping $p16^{INK4a}$ permanently repressed, thus facilitating transformation and development of leukemia. This might have implications in the rapid, yet debilitating progression of chronic phase to blast crisis stage of CML, where imatinib prognosis is poor (Wong and Witte, 2004), while the mechanisms that control the rate of disease progression remains unclear.

Moreover, a study has shown that imatinib treatment of CML was found to disturb bone remodeling, as patients show reduced bone formation (Berman *et al*.,2006). Thus, the possible toxicity of long-term imatinib mesylate therapy in humans have to be questioned, since it also inhibits the activity of c-Abl, whose deficient mice leads to senile osteoporosis, along with other defects. Therefore, long-term inhibition of abl kinase activity might lead to shortage of osteoblasts and osteopenia through the regulation of BMP activated Smad1/5/8 and MAPK pathways. Compounded by the ever increasing imatinib resistance in patients, and efforts to repress BCR-ABL transcripts or protein levels have led to limited progress due to the kinase-dependent nature CML pathogenesis (reviewed in O'Hare *et al*., 2007), perhaps targeting these key mediators may provide some relief to a long standing problem?

CHAPTER 10

REFERENCES

Abelson, H. T. and L. S. Rabstein (1970). "Lymphosarcoma: virus-induced thymic-independent disease in mice." Cancer Res **30**(8): 2213-22.

Akune, T., N. Ogata, K. Hoshi, N. Kubota, Y. Terauchi, K. Tobe, H. Takagi, Y. Azuma, T. Kadowaki, K. Nakamura and H. Kawaguchi (2002). "Insulin receptor substrate-2 maintains predominance of anabolic function over catabolic function of osteoblasts." J Cell Biol **159**(1): 147-56.

Alani, R. M., A. Z. Young and C. B. Shifflett (2001). "Id1 regulation of cellular senescence through transcriptional repression of p16/Ink4a." Proc Natl Acad Sci U S A **98**(14): 7812-6.

Avsian-Kretchmer, O. and A. J. Hsueh (2004). "Comparative genomic analysis of the eight-membered ring cystine knot-containing bone morphogenetic protein antagonists." Mol Endocrinol **18**(1): 1-12.

Bachiller, D., J. Klingensmith, C. Kemp, J. A. Belo, R. M. Anderson, S. R. May, J. A. McMahon, A. P. McMahon, R. M. Harland, J. Rossant and E. M. De Robertis (2000). "The organizer factors Chordin and Noggin are required for mouse forebrain development." Nature **403**(6770): 658-61.

Bachiller, D., J. Klingensmith, N. Shneyder, U. Tran, R. Anderson, J. Rossant and E. M. De Robertis (2003). "The role of chordin/Bmp signals in mammalian pharyngeal development and DiGeorge syndrome." Development **130**(15): 3567-78.

Beausejour, C. M., A. Krtolica, F. Galimi, M. Narita, S. W. Lowe, P. Yaswen and J. Campisi (2003). "Reversal of human cellular senescence: roles of the p53 and p16 pathways." EMBO J **22**(16): 4212-22.

Ben-Porath, I. and R. A. Weinberg (2004). "When cells get stressed: an integrative view of cellular senescence." J Clin Invest **113**(1): 8-13.

Benanti, J. A. and D. A. Galloway (2004). "Normal human fibroblasts are resistant to RAS-induced senescence." Mol Cell Biol **24**(7): 2842-52.

Benezra, R. (2001). "Role of Id proteins in embryonic and tumor angiogenesis." Trends Cardiovasc Med **11**(6): 237-41.

Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner and H. Weintraub (1990). "The protein Id: a negative regulator of helix-loop-helix DNA binding proteins." Cell **61**(1): 49-59.

Bennett, D. C. (2003). "Human melanocyte senescence and melanoma susceptibility genes." Oncogene **22**(20): 3063-9.

Beppu, H., M. Kawabata, T. Hamamoto, A. Chytil, O. Minowa, T. Noda and K. Miyazono (2000). "BMP type II receptor is required for gastrulation and early development of mouse embryos." Dev Biol **221**(1): 249-58.

Berman, E., M. Nicolaides, R. G. Maki, M. Fleisher, S. Chanel, K. Scheu, B. A. Wilson, G. Heller and N. P. Sauter (2006). "Altered bone and mineral metabolism in patients receiving imatinib mesylate." N Engl J Med **354**(19): 2006-13.

Bianco, P., M. Riminucci, S. Gronthos and P. G. Robey (2001). "Bone marrow stromal stem cells: nature, biology, and potential applications." Stem Cells **19**(3): 180-92.

Birkenkamp, K. U., A. Essafi, K. E. van der Vos, M. da Costa, R. C. Hui, F. Holstege, L. Koenderman, E. W. Lam and P. J. Coffer (2007). "FOXO3a induces differentiation of Bcr-Abl-transformed cells through transcriptional down-regulation of Id1." J Biol Chem **282**(4): 2211-20.

Bonyadi, M., S. D. Waldman, D. Liu, J. E. Aubin, M. D. Grynpas and W. L. Stanford (2003). "Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice." Proc Natl Acad Sci U S A **100**(10): 5840-5.

Boyden, L. M., J. Mao, J. Belsky, L. Mitzner, A. Farhi, M. A. Mitnick, D. Wu, K. Insogna and R. P. Lifton (2002). "High bone density due to a mutation in LDL-receptor-related protein 5." N Engl J Med **346**(20): 1513-21.

Braig, M., S. Lee, C. Loddenkemper, C. Rudolph, A. H. Peters, B. Schlegelberger, H. Stein, B. Dorken, T. Jenuwein and C. A. Schmitt (2005). "Oncogene-induced senescence as an initial barrier in lymphoma development." Nature **436**(7051): 660-5.

Brunet, L. J., J. A. McMahon, A. P. McMahon and R. M. Harland (1998). "Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton." Science **280**(5368): 1455-7.

Buckbinder, L., D. T. Crawford, H. Qi, H. Z. Ke, L. M. Olson, K. R. Long, P. C. Bonnette, A. P. Baumann, J. E. Hambor, W. A. Grasser, 3rd, L. C. Pan, T. A. Owen, M. J. Luzzio, C. A. Hulford, D. F. Gebhard, V. M. Paralkar, H. A. Simmons, J. C. Kath, W. G. Roberts, S. L. Smock, A. Guzman-Perez, T. A. Brown and M. Li (2007). "Proline-rich tyrosine kinase 2 regulates osteoprogenitor cells and bone formation, and offers an anabolic treatment approach for osteoporosis." Proc Natl Acad Sci U S A **104**(25): 10619-24.

Burkitt, H. G., B. Young and J. W. Heath (1999). " Wheater's Functional Histology. A Text and Colour Atlas." 3rd Edition. Churchill Livingstone.

Calvi, L. M. (2006). "Osteoblastic activation in the hematopoietic stem cell niche." Ann N Y Acad Sci **1068**: 477-88.

Calvi, L. M., G. B. Adams, K. W. Weibrecht, J. M. Weber, D. P. Olson, M. C. Knight, R. P. Martin, E. Schipani, P. Divieti, F. R. Bringhurst, L. A. Milner, H. M. Kronenberg and D. T. Scadden (2003). "Osteoblastic cells regulate the haematopoietic stem cell niche." Nature **425**(6960): 841-6.

Cammarano, M. S., T. Nekrasova, B. Noel and A. Minden (2005). "Pak4 induces premature senescence via a pathway requiring p16INK4/p19ARF and mitogen-activated protein kinase signaling." Mol Cell Biol **25**(21): 9532-42.

Campisi, J. (1996). "Replicative senescence: an old lives' tale?" Cell **84**(4): 497-500.

Campisi, J. (1997). "The biology of replicative senescence." Eur J Cancer **33**(5): 703-9.

Campisi, J. (2001). "From cells to organisms: can we learn about aging from cells in culture?" Exp Gerontol **36**(4-6): 607-18.

Campisi, J. (2005). "Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors." Cell **120**(4): 513-22.

Canalis, E., A. Giustina and J. P. Bilezikian (2007). "Mechanisms of anabolic therapies for osteoporosis." N Engl J Med **357**(9): 905-16.

Cao, C., Y. Leng and D. Kufe (2003). "Catalase activity is regulated by c-Abl and Arg in the oxidative stress response." J Biol Chem **278**(32): 29667-75.

Carmeliet, P. (1999). "Developmental biology. Controlling the cellular brakes." Nature **401**(6754): 657-8.

Carnero, A., J. D. Hudson, C. M. Price and D. H. Beach (2000). "p16INK4A and p19ARF act in overlapping pathways in cellular immortalization." Nat Cell Biol **2**(3): 148-55.

Chan, G. K. and G. Duque (2002). "Age-related bone loss: old bone, new facts." Gerontology **48**(2): 62-71.

Chang, S., A. S. Multani, N. G. Cabrera, M. L. Naylor, P. Laud, D. Lombard, S. Pathak, L. Guarente and R. A. DePinho (2004). "Essential role of limiting telomeres in the pathogenesis of Werner syndrome." Nat Genet **36**(8): 877-82.

Chen, D., M. Zhao, S. E. Harris and Z. Mi (2004). "Signal transduction and biological functions of bone morphogenetic proteins." Front Biosci **9**: 349-58.

Chen, G., S. S. Yuan, W. Liu, Y. Xu, K. Trujillo, B. Song, F. Cong, S. P. Goff, Y. Wu, R. Arlinghaus, D. Baltimore, P. J. Gasser, M. S. Park, P. Sung and E. Y. Lee (1999). "Radiation-induced assembly of Rad51 and Rad52 recombination complex requires ATM and c-Abl." J Biol Chem **274**(18): 12748-52.

Chen, H. B., J. Shen, Y. T. Ip and L. Xu (2006). "Identification of phosphatases for Smad in the BMP/DPP pathway." Genes Dev **20**(6): 648-53.

Choo, M. K., N. Kawasaki, P. Singhirunnusorn, K. Koizumi, S. Sato, S. Akira, I. Saiki and H. Sakurai (2006). "Blockade of transforming growth factor-beta-activated kinase 1 activity enhances TRAIL-induced apoptosis through activation of a caspase cascade." Mol Cancer Ther **5**(12): 2970-6.

Cohen, M. H., J. R. Johnson and R. Pazdur (2005). "U.S. Food and Drug Administration Drug Approval Summary: conversion of imatinib mesylate (STI571; Gleevec) tablets from accelerated approval to full approval." Clin Cancer Res **11**(1): 12-9.

Daniels, C. E., M. C. Wilkes, M. Edens, T. J. Kottom, S. J. Murphy, A. H. Limper and E. B. Leof (2004). "Imatinib mesylate inhibits the profibrogenic activity of TGF-beta and prevents bleomycin-mediated lung fibrosis." J Clin Invest **114**(9): 1308-16.

Deininger, M. W., S. Vieira, R. Mendiola, B. Schultheis, J. M. Goldman and J. V. Melo (2000). "BCR-ABL tyrosine kinase activity regulates the expression of multiple genes implicated in the pathogenesis of chronic myeloid leukemia." Cancer Res **60**(7): 2049-55.

Delaney, J. R. and M. Mlodzik (2006). "TGF-beta activated kinase-1: new insights into the diverse roles of TAK1 in development and immunity." Cell Cycle **5**(24): 2852-5.

Deng, Z., J. H. Morse, S. L. Slager, N. Cuervo, K. J. Moore, G. Venetos, S. Kalachikov, E. Cayanis, S. G. Fischer, R. J. Barst, S. E. Hodge and J. A. Knowles (2000). "Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene." Am J Hum Genet **67**(3): 737-44.

Dierov, J., R. Dierova and M. Carroll (2004). "BCR/ABL translocates to the nucleus and disrupts an ATR-dependent intra-S phase checkpoint." Cancer Cell **5**(3): 275-85.

Dimri, G. P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith and et al. (1995). "A biomarker that identifies senescent human cells in culture and in aging skin in vivo." Proc Natl Acad Sci U S A **92**(20): 9363-7.

Druker, B. J., S. Tamura, E. Buchdunger, S. Ohno, G. M. Segal, S. Fanning, J. Zimmermann and N. B. Lydon (1996). "Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells." Nat Med **2**(5): 561-6.

Duan, X., Y. Y. Liang, X. H. Feng and X. Lin (2006). "Protein serine/threonine phosphatase PPM1A dephosphorylates Smad1 in the bone morphogenetic protein signaling pathway." J Biol Chem **281**(48): 36526-32.

Ducy, P., T. Schinke and G. Karsenty (2000). "The osteoblast: a sophisticated fibroblast under central surveillance." Science **289**(5484): 1501-4.

Ducy, P., R. Zhang, V. Geoffroy, A. L. Ridall and G. Karsenty (1997). "Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation." Cell **89**(5): 747-54.

Feller, S. M. (2001). "Crk family adaptors-signalling complex formation and biological roles." Oncogene **20**(44): 6348-71.

Foletta, V. C., M. A. Lim, J. Soosairajah, A. P. Kelly, E. G. Stanley, M. Shannon, W. He, S. Das, J. Massague and O. Bernard (2003). "Direct signaling by the BMP type II receptor via the cytoskeletal regulator LIMK1." J Cell Biol **162**(6): 1089-98.

Fowlkes, J. L., K. M. Thrailkill, L. Liu, E. C. Wahl, R. C. Bunn, G. E. Cockrell, D. S. Perrien, J. Aronson and C. K. Lumpkin, Jr. (2006). "Effects of systemic and local administration of recombinant human IGF-I (rhIGF-I) on de novo bone formation in an aged mouse model." J Bone Miner Res **21**(9): 1359-66.

Gannon, F. H., F. S. Kaplan, E. Olmsted, G. C. Finkel, M. A. Zasloff and E. Shore (1997). "Bone morphogenetic protein 2/4 in early fibromatous lesions of fibrodysplasia ossificans progressiva." Hum Pathol **28**(3): 339-43.

Gesbert, F., W. R. Sellers, S. Signoretti, M. Loda and J. D. Griffin (2000). "BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor p27Kip1 through the phosphatidylinositol 3-Kinase/AKT pathway." J Biol Chem **275**(50): 39223-30.

Gire, V. and D. Wynford-Thomas (1998). "Reinitiation of DNA synthesis and cell division in senescent human fibroblasts by microinjection of anti-p53 antibodies." Mol Cell Biol **18**(3): 1611-21.

Goldman, R. D., D. K. Shumaker, M. R. Erdos, M. Eriksson, A. E. Goldman, L. B. Gordon, Y. Gruenbaum, S. Khuon, M. Mendez, R. Varga and F. S. Collins (2004). "Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome." Proc Natl Acad Sci U S A **101**(24): 8963-8.

Gong, Y., D. Krakow, J. Marcelino, D. Wilkin, D. Chitayat, R. Babul-Hirji, L. Hudgins, C. W. Cremers, F. P. Cremers, H. G. Brunner, K. Reinker, D. L. Rimoin, D. H. Cohn, F. R. Goodman, W. Reardon, M. Patton, C. A. Francomano and M. L. Warman (1999). "Heterozygous mutations in the gene encoding noggin affect human joint morphogenesis." Nat Genet **21**(3): 302-4.

Groffen, J., J. R. Stephenson, N. Heisterkamp, A. de Klein, C. R. Bartram and G. Grosveld (1984). "Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22." Cell **36**(1): 93-9.

Guyton, A. C., and J. E. Hall (1996). "Textbook of Medical Physiology." 9th Edition, WB Saunders Company.

Hadjidakis, D. J. and Androulakis, II (2006). "Bone remodeling." Ann N Y Acad Sci **1092**: 385-96.

Hammaker, D. R., D. L. Boyle, M. Chabaud-Riou and G. S. Firestein (2004). "Regulation of c-Jun N-terminal kinase by MEKK-2 and mitogen-activated protein kinase kinase kinases in rheumatoid arthritis." J Immunol **172**(3): 1612-8.

Hantschel, O. and G. Superti-Furga (2004). "Regulation of the c-Abl and Bcr-Abl tyrosine kinases." Nat Rev Mol Cell Biol **5**(1): 33-44.

Hara, E., H. Tsurui, A. Shinozaki, S. Nakada and K. Oda (1991). "Cooperative effect of antisense-Rb and antisense-p53 oligomers on the extension of life span in human diploid fibroblasts, TIG-1." Biochem Biophys Res Commun **179**(1): 528-34.

Harrison, S. C. (2003). "Variation on an Src-like theme." Cell **112**(6): 737-40.

Hartung, A., K. Bitton-Worms, M. M. Rechtman, V. Wenzel, J. H. Boergermann, S. Hassel, Y. I. Henis and P. Knaus (2006). "Different routes of bone morphogenic protein (BMP) receptor endocytosis influence BMP signaling." Mol Cell Biol **26**(20): 7791-805.

Hassel, S., M. Yakymovych, U. Hellman, L. Ronnstrand, P. Knaus and S. Souchelnytskyi (2006). "Interaction and functional cooperation between the serine/threonine kinase bone morphogenetic protein type II receptor with the tyrosine kinase stem cell factor receptor." J Cell Physiol **206**(2): 457-67.

Hasty, P., J. Campisi, J. Hoeijmakers, H. van Steeg and J. Vijg (2003). "Aging and genome maintenance: lessons from the mouse?" Science **299**(5611): 1355-9.

Hay, E., J. Lemonnier, O. Fromigue and P. J. Marie (2001). "Bone morphogenetic protein-2 promotes osteoblast apoptosis through a Smad-independent, protein kinase C-dependent signaling pathway." J Biol Chem **276**(31): 29028-36.

Hayflick, L. and P. S. Moorhead (1961). "The serial cultivation of human diploid cell strains." Exp Cell Res **25**: 585-621.

Heisterkamp, N., K. Stam, J. Groffen, A. de Klein and G. Grosveld (1985). "Structural organization of the bcr gene and its role in the Ph' translocation." Nature **315**(6022): 758-61.

Hishiya, A., M. Ito, H. Aburatani, N. Motoyama, K. Ikeda and K. Watanabe (2005). "Ataxia telangiectasia mutated (Atm) knockout mice as a model of osteopenia due to impaired bone formation." Bone **37**(4): 497-503.

Hoffmann, A., O. Preobrazhenska, C. Wodarczyk, Y. Medler, A. Winkel, S. Shahab, D. Huylebroeck, G. Gross and K. Verschueren (2005). "Transforming growth factor-beta-activated kinase-1 (TAK1), a MAP3K, interacts with Smad proteins and interferes with osteogenesis in murine mesenchymal progenitors." J Biol Chem **280**(29): 27271-83.

Hogan, B. L. (1996). "Bone morphogenetic proteins: multifunctional regulators of vertebrate development." Genes Dev **10**(13): 1580-94.

Holst, C. R., G. J. Nuovo, M. Esteller, K. Chew, S. B. Baylin, J. G. Herman and T. D. Tlsty (2003). "Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia." Cancer Res **63**(7): 1596-601.

Huot, T. J., J. Rowe, M. Harland, S. Drayton, S. Brookes, C. Gooptu, P. Purkis, M. Fried, V. Bataille, E. Hara, J. Newton-Bishop and G. Peters (2002). "Biallelic mutations in p16(INK4a) confer resistance to Ras- and Ets-induced senescence in human diploid fibroblasts." Mol Cell Biol **22**(23): 8135-43.

Ishiguro, A., K. S. Spirin, M. Shiohara, A. Tobler, A. F. Gombart, M. A. Israel, J. D. Norton and H. P. Koeffler (1996). "Id2 expression increases with differentiation of human myeloid cells." Blood **87**(12): 5225-31.

Itahana, K., J. Campisi and G. P. Dimri (2004). "Mechanisms of cellular senescence in human and mouse cells." Biogerontology **5**(1): 1-10.

Itahana, K., Y. Zou, Y. Itahana, J. L. Martinez, C. Beausejour, J. J. Jacobs, M. Van Lohuizen, V. Band, J. Campisi and G. P. Dimri (2003). "Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1." Mol Cell Biol **23**(1): 389-401.

Jadrich, J. L., M. B. O'Connor and E. Coucouvanis (2003). "Expression of TAK1, a mediator of TGF-beta and BMP signaling, during mouse embryonic development." Gene Expr Patterns **3**(2): 131-4.

Janzen, V., R. Forkert, H. E. Fleming, Y. Saito, M. T. Waring, D. M. Dombkowski, T. Cheng, R. A. DePinho, N. E. Sharpless and D. T. Scadden (2006). "Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a." Nature **443**(7110): 421-6.

Jen, Y., K. Manova and R. Benezra (1997). "Each member of the Id gene family exhibits a unique expression pattern in mouse gastrulation and neurogenesis." Dev Dyn **208**(1): 92-106.

Jen, Y., H. Weintraub and R. Benezra (1992). "Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins." Genes Dev **6**(8): 1466-79.

Jilka, R. L., R. S. Weinstein, K. Takahashi, A. M. Parfitt and S. C. Manolagas (1996). "Linkage of decreased bone mass with impaired osteoblastogenesis in a murine model of accelerated senescence." J Clin Invest **97**(7): 1732-40.

Jin, W., C. Yun, H. S. Kim and S. J. Kim (2007). "TrkC binds to the bone morphogenetic protein type II receptor to suppress bone morphogenetic protein signaling." Cancer Res **67**(20): 9869-77.

Kadowaki, T., K. Tobe, R. Honda-Yamamoto, H. Tamemoto, Y. Kaburagi, K. Momomura, K. Ueki, Y. Takahashi, T. Yamauchi, Y. Akanuma and Y. Yazaki (1996). "Signal transduction mechanism of insulin and insulin-like growth factor-1." Endocr J **43 Suppl**: S33-41.

Kaps, C., A. Hoffmann, Y. Zilberman, G. Pelled, T. Haupl, M. Sittinger, G. Burmester, D. Gazit and G. Gross (2004). "Distinct roles of BMP receptors Type IA

and IB in osteo-/chondrogenic differentiation in mesenchymal progenitors (C3H10T1/2)." Biofactors **20**(2): 71-84.

Kato, M., M. S. Patel, R. Levasseur, I. Lobov, B. H. Chang, D. A. Glass, 2nd, C. Hartmann, L. Li, T. H. Hwang, C. F. Brayton, R. A. Lang, G. Karsenty and L. Chan (2002). "Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor." J Cell Biol **157**(2): 303-14.

Kawabata, M., T. Imamura and K. Miyazono (1998). "Signal transduction by bone morphogenetic proteins." Cytokine Growth Factor Rev **9**(1): 49-61.

Kawaguchi, H. (2006). "Molecular backgrounds of age-related osteoporosis from mouse genetics approaches." Rev Endocr Metab Disord **7**(1-2): 17-22.

Kawaguchi, H., N. Manabe, C. Miyaura, H. Chikuda, K. Nakamura and M. Kuro-o (1999). "Independent impairment of osteoblast and osteoclast differentiation in klotho mouse exhibiting low-turnover osteopenia." J Clin Invest **104**(3): 229-37.

Kendall, S. E., C. Battelli, S. Irwin, J. G. Mitchell, C. A. Glackin and J. M. Verdi (2005). "NRAGE mediates p38 activation and neural progenitor apoptosis via the bone morphogenetic protein signaling cascade." Mol Cell Biol **25**(17): 7711-24.

Kim, H. J., J. H. Kim, S. C. Bae, J. Y. Choi and H. M. Ryoo (2003). "The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2." J Biol Chem **278**(1): 319-26.

Kim, J. H., G. E. Lee, J. C. Kim, J. H. Lee and I. K. Chung (2002). "A novel telomere elongation in an adriamycin-resistant stomach cancer cell line with decreased telomerase activity." Mol Cells **13**(2): 228-36.

Kim, W. Y. and N. E. Sharpless (2006). "The regulation of INK4/ARF in cancer and aging." Cell **127**(2): 265-75.

Kipling, D., T. Davis, E. L. Ostler and R. G. Faragher (2004). "What can progeroid syndromes tell us about human aging?" Science **305**(5689): 1426-31.

Kirkwood, T. B. (1977). "Evolution of ageing." Nature **270**(5635): 301-4.

Kirkwood, T. B. (2005). "Understanding the odd science of aging." Cell **120**(4): 437-47.

Kirkwood, T. B. and S. N. Austad (2000). "Why do we age?" Nature **408**(6809): 233-8.

Knockaert, M., G. Sapkota, C. Alarcon, J. Massague and A. H. Brivanlou (2006). "Unique players in the BMP pathway: small C-terminal domain phosphatases dephosphorylate Smad1 to attenuate BMP signaling." Proc Natl Acad Sci U S A **103**(32): 11940-5.

Kohmura, K., Y. Miyakawa, Y. Kawai, Y. Ikeda and M. Kizaki (2004). "Different roles of p38 MAPK and ERK in STI571-induced multi-lineage differentiation of K562 cells." J Cell Physiol **198**(3): 370-6.

Koleske, A. J., A. M. Gifford, M. L. Scott, M. Nee, R. T. Bronson, K. A. Miczek and D. Baltimore (1998). "Essential roles for the Abl and Arg tyrosine kinases in neurulation." Neuron **21**(6): 1259-72.

Komori, T., H. Yagi, S. Nomura, A. Yamaguchi, K. Sasaki, K. Deguchi, Y. Shimizu, R. T. Bronson, Y. H. Gao, M. Inada, M. Sato, R. Okamoto, Y. Kitamura, S. Yoshiki and T. Kishimoto (1997). "Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts." Cell **89**(5): 755-64.

Korchynskyi, O. and P. ten Dijke (2002). "Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter." J Biol Chem **277**(7): 4883-91.

Krause, D. S. and R. A. Van Etten (2005). "Tyrosine kinases as targets for cancer therapy." N Engl J Med **353**(2): 172-87.

Kreider, B. L., R. Benezra, G. Rovera and T. Kadesch (1992). "Inhibition of myeloid differentiation by the helix-loop-helix protein Id." Science **255**(5052): 1700-2.

Krishnamurthy, J., M. R. Ramsey, K. L. Ligon, C. Torrice, A. Koh, S. Bonner-Weir and N. E. Sharpless (2006). "p16INK4a induces an age-dependent decline in islet regenerative potential." Nature **443**(7110): 453-7.

Krishnamurthy, J., C. Torrice, M. R. Ramsey, G. I. Kovalev, K. Al-Regaiey, L. Su and N. E. Sharpless (2004). "Ink4a/Arf expression is a biomarker of aging." J Clin Invest **114**(9): 1299-307.

Kronenberg, H. M. (2003). "Developmental regulation of the growth plate." Nature **423**(6937): 332-6.

Kruh, G. D., R. Perego, T. Miki and S. A. Aaronson (1990). "The complete coding sequence of arg defines the Abelson subfamily of cytoplasmic tyrosine kinases." Proc Natl Acad Sci U S A **87**(15): 5802-6.

Kuribara, R., H. Honda, H. Matsui, T. Shinjyo, T. Inukai, K. Sugita, S. Nakazawa, H. Hirai, K. Ozawa and T. Inaba (2004). "Roles of Bim in apoptosis of normal and Bcr-Abl-expressing hematopoietic progenitors." Mol Cell Biol **24**(14): 6172-83.

Lane, K. B., R. D. Machado, M. W. Pauciulo, J. R. Thomson, J. A. Phillips, 3rd, J. E. Loyd, W. C. Nichols and R. C. Trembath (2000). "Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium." Nat Genet **26**(1): 81-4.

Lazner, F., M. Gowen, D. Pavasovic and I. Kola (1999). "Osteopetrosis and osteoporosis: two sides of the same coin." Hum Mol Genet **8**(10): 1839-46.

Lee, J., M. J. Son, K. Woolard, N. M. Donin, A. Li, C. H. Cheng, S. Kotliarova, Y. Kotliarov, J. Walling, S. Ahn, M. Kim, M. Totonchy, T. Cusack, C. Ene, H. Ma, Q. Su, J. C. Zenklusen, W. Zhang, D. Maric and H. A. Fine (2008). "Epigenetic-mediated dysfunction of the bone morphogenetic protein pathway inhibits differentiation of glioblastoma-initiating cells." Cancer Cell **13**(1): 69-80.

Lee, K. S., H. J. Kim, Q. L. Li, X. Z. Chi, C. Ueta, T. Komori, J. M. Wozney, E. G. Kim, J. Y. Choi, H. M. Ryoo and S. C. Bae (2000). "Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12." Mol Cell Biol **20**(23): 8783-92.

Li, B., S. Boast, K. de los Santos, I. Schieren, M. Quiroz, S. L. Teitelbaum, M. M. Tondravi and S. P. Goff (2000). "Mice deficient in Abl are osteoporotic and have defects in osteoblast maturation." Nat Genet **24**(3): 304-8.

Li, B., X. Wang, N. Rasheed, Y. Hu, S. Boast, T. Ishii, K. Nakayama, K.I. Nakayama, S.P. Goff (2004). " Distinct roles of c-Abl and Atm in oxidative stress response are mediated by protein kinase C delta." Genes Dev **18** (15) : 1824-37.

Li, B. (2006). " Inhibition of Abl Kinases by Intramolecular and Intermolecular Interactions: A Lesson from Structure Studies and CML Therapy." Current Enzyme Inhibition **2**: 135-146

Liang, M., G. Russell and P. A. Hulley (2008). "Bim, Bak, and Bax regulate osteoblast survival." J Bone Miner Res **23**(5): 610-20.

Lin, A. W., M. Barradas, J. C. Stone, L. van Aelst, M. Serrano and S. W. Lowe (1998). "Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling." Genes Dev **12**(19): 3008-19.

Lin, X., X. Duan, Y. Y. Liang, Y. Su, K. H. Wrighton, J. Long, M. Hu, C. M. Davis, J. Wang, F. C. Brunicardi, Y. Shi, Y. G. Chen, A. Meng and X. H. Feng (2006). "PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling." Cell **125**(5): 915-28.

Lin, X., Y. Y. Liang, B. Sun, M. Liang, Y. Shi, F. C. Brunicardi and X. H. Feng (2003). "Smad6 recruits transcription corepressor CtBP to repress bone morphogenetic protein-induced transcription." Mol Cell Biol **23**(24): 9081-93.

Logothetis, C. J. and S. H. Lin (2005). "Osteoblasts in prostate cancer metastasis to bone." Nat Rev Cancer **5**(1): 21-8.

Lombard, D. B., K. F. Chua, R. Mostoslavsky, S. Franco, M. Gostissa and F. W. Alt (2005). "DNA repair, genome stability, and aging." Cell **120**(4): 497-512.

Lopez-Rovira, T., E. Chalaux, J. Massague, J. L. Rosa and F. Ventura (2002). "Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene." J Biol Chem **277**(5): 3176-85.

Lowe, S. W. and C. J. Sherr (2003). "Tumor suppression by Ink4a-Arf: progress and puzzles." Curr Opin Genet Dev **13**(1): 77-83.

Lundberg, A. S., W. C. Hahn, P. Gupta and R. A. Weinberg (2000). "Genes involved in senescence and immortalization." Curr Opin Cell Biol **12**(6): 705-9.

Lyden, D., A. Z. Young, D. Zagzag, W. Yan, W. Gerald, R. O'Reilly, B. L. Bader, R. O. Hynes, Y. Zhuang, K. Manova and R. Benezra (1999). "Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts." Nature **401**(6754): 670-7.

Maeda, Y., K. Tsuji, A. Nifuji and M. Noda (2004). "Inhibitory helix-loop-helix transcription factors Id1/Id3 promote bone formation in vivo." J Cell Biochem **93**(2): 337-44.

Manolagas, S. C. (2000). "Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis." Endocr Rev **21**(2): 115-37.

Manolagas, S. C. and R. L. Jilka (1995). "Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis." N Engl J Med **332**(5): 305-11.

Martin, G. M. (1985). "Genetics and aging; the Werner syndrome as a segmental progeroid syndrome." Adv Exp Med Biol **190**: 161-70.

Massague, J. (1996). "TGFbeta signaling: receptors, transducers, and Mad proteins." Cell **85**(7): 947-50.

Massague, J. (2000). "How cells read TGF-beta signals." Nat Rev Mol Cell Biol **1**(3): 169-78.

Massague, J. (2003). "Integration of Smad and MAPK pathways: a link and a linker revisited." Genes Dev **17**(24): 2993-7.

Massague, J., L. Attisano and J. L. Wrana (1994). "The TGF-beta family and its composite receptors." Trends Cell Biol **4**(5): 172-8.

Massari, M. E. and C. Murre (2000). "Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms." Mol Cell Biol **20**(2): 429-40.

Matheu, A., C. Pantoja, A. Efeyan, L. M. Criado, J. Martin-Caballero, J. M. Flores, P. Klatt and M. Serrano (2004). "Increased gene dosage of Ink4a/Arf results in cancer resistance and normal aging." Genes Dev **18**(22): 2736-46.

Mathew, S., W. Chen, V. V. Murty, R. Benezra and R. S. Chaganti (1995). "Chromosomal assignment of human ID1 and ID2 genes." Genomics **30**(2): 385-7.

Mathon, N. F. and A. C. Lloyd (2001). "Cell senescence and cancer." Nat Rev Cancer **1**(3): 203-13.

Matsushita, M., T. Tsuboyama, R. Kasai, H. Okumura, T. Yamamuro, K. Higuchi, A. Kohno, T. Yonezu, A. Utani and et al. (1986). "Age-related changes in bone mass in the senescence-accelerated mouse (SAM). SAM-R/3 and SAM-P/6 as new murine models for senile osteoporosis." Am J Pathol **125**(2): 276-83.

Melo, J. V. (1996). "The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype." Blood **88**(7): 2375-84.

Migliaccio, E., M. Giorgio, S. Mele, G. Pelicci, P. Reboldi, P. P. Pandolfi, L. Lanfrancone and P. G. Pelicci (1999). "The p66shc adaptor protein controls oxidative stress response and life span in mammals." Nature **402**(6759): 309-13.

Million, R. P. and R. A. Van Etten (2000). "The Grb2 binding site is required for the induction of chronic myeloid leukemia-like disease in mice by the Bcr/Abl tyrosine kinase." Blood **96**(2): 664-70.

Millis, A. J., M. Hoyle, H. M. McCue and H. Martini (1992). "Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase genes in aged human fibroblasts." Exp Cell Res **201**(2): 373-9.

Mishina, Y., A. Suzuki, N. Ueno and R. R. Behringer (1995). "Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis." Genes Dev **9**(24): 3027-37.

Miyazono, K., K. Kusanagi and H. Inoue (2001). "Divergence and convergence of TGF-beta/BMP signaling." J Cell Physiol **187**(3): 265-76.

Miyazono, K., S. Maeda and T. Imamura (2005). "BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk." Cytokine Growth Factor Rev **16**(3): 251-63.

Miyazono, K. and K. Miyazawa (2002). "Id: a target of BMP signaling." Sci STKE **2002**(151): PE40.

Molofsky, A. V., S. G. Slutsky, N. M. Joseph, S. He, R. Pardal, J. Krishnamurthy, N. E. Sharpless and S. J. Morrison (2006). "Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing." Nature **443**(7110): 448-52.

Moriguchi, T., N. Kuroyanagi, K. Yamaguchi, Y. Gotoh, K. Irie, T. Kano, K. Shirakabe, Y. Muro, H. Shibuya, K. Matsumoto, E. Nishida and M. Hagiwara (1996). "A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3." J Biol Chem **271**(23): 13675-9.

Morrison, S. J., A. M. Wandycz, K. Akashi, A. Globerson and I. L. Weissman (1996). "The aging of hematopoietic stem cells." Nat Med **2**(9): 1011-6.

Muller, R., D. J. Slamon, J. M. Tremblay, M. J. Cline and I. M. Verma (1982). "Differential expression of cellular oncogenes during pre- and postnatal development of the mouse." Nature **299**(5884): 640-4.

Mundy, G. R., D. Chen, M. Zhao, S. Dallas, C. Xu and S. Harris (2001). "Growth regulatory factors and bone." Rev Endocr Metab Disord **2**(1): 105-15.

Nagar, B., O. Hantschel, M. A. Young, K. Scheffzek, D. Veach, W. Bornmann, B. Clarkson, G. Superti-Furga and J. Kuriyan (2003). "Structural basis for the autoinhibition of c-Abl tyrosine kinase." Cell **112**(6): 859-71.

Nakamura, Y., T. Yujiri, R. Nawata, K. Tagami and Y. Tanizawa (2005). "MEK kinase 1 is essential for Bcr-Abl-induced STAT3 and self-renewal activity in embryonic stem cells." Oncogene **24**(51): 7592-8.

Nakashima, K., M. Yanagisawa, H. Arakawa, N. Kimura, T. Hisatsune, M. Kawabata, K. Miyazono and T. Taga (1999). "Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300." Science **284**(5413): 479-82.

Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J. M. Deng, R. R. Behringer and B. de Crombrugghe (2002). "The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation." Cell **108**(1): 17-29.

Namiki, M., S. Akiyama, T. Katagiri, A. Suzuki, N. Ueno, N. Yamaji, V. Rosen, J. M. Wozney and T. Suda (1997). "A kinase domain-truncated type I receptor blocks bone morphogenetic protein-2-induced signal transduction in C2C12 myoblasts." J Biol Chem **272**(35): 22046-52.

Narita, M., V. Krizhanovsky, S. Nunez, A. Chicas, S. A. Hearn, M. P. Myers and S. W. Lowe (2006). "A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation." Cell **126**(3): 503-14.

Narita, M. and S. W. Lowe (2005). "Senescence comes of age." Nat Med **11**(9): 920-2.

Narita, M., S. Nunez, E. Heard, A. W. Lin, S. A. Hearn, D. L. Spector, G. J. Hannon and S. W. Lowe (2003). "Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence." Cell **113**(6): 703-16.

Naski, M. C. and D. M. Ornitz (1998). "FGF signaling in skeletal development." Front Biosci **3**: d781-94.

Nieborowska-Skorska, M., G. Hoser, L. Rink, M. Malecki, P. Kossev, M. A. Wasik and T. Skorski (2006). "Id1 transcription inhibitor-matrix metalloproteinase 9 axis enhances invasiveness of the breakpoint cluster region/abelson tyrosine kinase-transformed leukemia cells." Cancer Res **66**(8): 4108-16.

Nielsen, G. P., A. O. Stemmer-Rachamimov, J. Shaw, J. E. Roy, J. Koh and D. N. Louis (1999). "Immunohistochemical survey of p16INK4A expression in normal human adult and infant tissues." Lab Invest **79**(9): 1137-43.

Ninomiya-Tsuji, J., K. Kishimoto, A. Hiyama, J. Inoue, Z. Cao and K. Matsumoto (1999). "The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway." Nature **398**(6724): 252-6.

Nishihara, A., T. Watabe, T. Imamura and K. Miyazono (2002). "Functional heterogeneity of bone morphogenetic protein receptor-II mutants found in patients with primary pulmonary hypertension." Mol Biol Cell **13**(9): 3055-63.

Nohe, A., S. Hassel, M. Ehrlich, F. Neubauer, W. Sebald, Y. I. Henis and P. Knaus (2002). "The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways." J Biol Chem **277**(7): 5330-8.

Nohe, A., E. Keating, P. Knaus and N. O. Petersen (2004). "Signal transduction of bone morphogenetic protein receptors." Cell Signal **16**(3): 291-9.

Nohe, A., E. Keating, T. M. Underhill, P. Knaus and N. O. Petersen (2003). "Effect of the distribution and clustering of the type I A BMP receptor (ALK3) with the type II BMP receptor on the activation of signalling pathways." J Cell Sci **116**(Pt 16): 3277-84.

Norton, J. D., R. W. Deed, G. Craggs and F. Sablitzky (1998). "Id helix-loop-helix proteins in cell growth and differentiation." Trends Cell Biol **8**(2): 58-65.

Nunes, I., R. D. Higgins, L. Zanetta, P. Shamamian and S. P. Goff (2001). "c-abl is required for the development of hyperoxia-induced retinopathy." J Exp Med **193**(12): 1383-91.

O'Brien, S. G. and M. W. Deininger (2003). "Imatinib in patients with newly diagnosed chronic-phase chronic myeloid leukemia." Semin Hematol **40**(2 Suppl 2): 26-30.

O'Hare, T., C. A. Eide and M. W. Deininger (2007). "Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia." Blood **110**(7): 2242-9.

O'Neill, A. J., T. G. Cotter, J. M. Russell and E. F. Gaffney (1997). "Abl expression in human fetal and adult tissues, tumours, and tumour microvessels." J Pathol **183**(3): 325-9.

Ogata, N., D. Chikazu, N. Kubota, Y. Terauchi, K. Tobe, Y. Azuma, T. Ohta, T. Kadowaki, K. Nakamura and H. Kawaguchi (2000). "Insulin receptor substrate-1 in osteoblast is indispensable for maintaining bone turnover." J Clin Invest **105**(7): 935-43.

Ohtani, N., K. Yamakoshi, A. Takahashi and E. Hara (2004). "The p16INK4a-RB pathway: molecular link between cellular senescence and tumor suppression." J Med Invest **51**(3-4): 146-53.

Ohtani, N., Z. Zebedee, T. J. Huot, J. A. Stinson, M. Sugimoto, Y. Ohashi, A. D. Sharrocks, G. Peters and E. Hara (2001). "Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence." Nature **409**(6823): 1067-70.

Okamoto, A., D. J. Demetrick, E. A. Spillare, K. Hagiwara, S. P. Hussain, W. P. Bennett, K. Forrester, B. Gerwin, M. Serrano, D. H. Beach and et al. (1994).

"Mutations and altered expression of p16INK4 in human cancer." Proc Natl Acad Sci U S A **91**(23): 11045-9.

Okuda, K., E. Weisberg, D. G. Gilliland and J. D. Griffin (2001). "ARG tyrosine kinase activity is inhibited by STI571." Blood **97**(8): 2440-8.

Onichtchouk, D., Y. G. Chen, R. Dosch, V. Gawantka, H. Delius, J. Massague and C. Niehrs (1999). "Silencing of TGF-beta signalling by the pseudoreceptor BAMBI." Nature **401**(6752): 480-5.

Osses, N., J. Gutierrez, T. Lopez-Rovira, F. Ventura and E. Brandan (2006). "Sulfation is required for bone morphogenetic protein 2-dependent Id1 induction." Biochem Biophys Res Commun **344**(4): 1207-15.

Osyczka, A. M. and P. S. Leboy (2005). "Bone morphogenetic protein regulation of early osteoblast genes in human marrow stromal cells is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase signaling." Endocrinology **146**(8): 3428-37.

Palmero, I., C. Pantoja and M. Serrano (1998). "p19ARF links the tumour suppressor p53 to Ras." Nature **395**(6698): 125-6.

Partridge, L. and D. Gems (2002). "The evolution of longevity." Curr Biol **12**(16): R544-6.

Passegue, E., E. F. Wagner and I. L. Weissman (2004). "JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells." Cell **119**(3): 431-43.

Pendergast, A. M. (2002). "The Abl family kinases: mechanisms of regulation and signaling." Adv Cancer Res **85**: 51-100.

Pera, E. M., A. Ikeda, E. Eivers and E. M. De Robertis (2003). "Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction." Genes Dev **17**(24): 3023-8.

Perk, J., A. Iavarone and R. Benezra (2005). "Id family of helix-loop-helix proteins in cancer." Nat Rev Cancer **5**(8): 603-14.

Perry, S. S., Y. Zhao, L. Nie, S. W. Cochrane, Z. Huang and X. H. Sun (2007). "Id1, but not Id3, directs long-term repopulating hematopoietic stem-cell maintenance." Blood **110**(7): 2351-60.

Piccolo, S., Y. Sasai, B. Lu and E. M. De Robertis (1996). "Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4." Cell **86**(4): 589-98.

Plattner, R., B. J. Irvin, S. Guo, K. Blackburn, A. Kazlauskas, R. T. Abraham, J. D. York and A. M. Pendergast (2003). "A new link between the c-Abl tyrosine kinase and phosphoinositide signalling through PLC-gamma1." Nat Cell Biol **5**(4): 309-19.

Plattner, R., L. Kadlec, K. A. DeMali, A. Kazlauskas and A. M. Pendergast (1999). "c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF." Genes Dev **13**(18): 2400-11.

Pogoda, P., M. Priemel, A. F. Schilling, M. Gebauer, P. Catala-Lehnen, F. Barvencik, F. T. Beil, C. Munch, M. Rupprecht, C. Muldner, J. M. Rueger, T. Schinke and M. Amling (2005). "Mouse models in skeletal physiology and osteoporosis: experiences and data on 14,839 cases from the Hamburg Mouse Archives." J Bone Miner Metab **23 Suppl**: 97-102.

Qi, X., T. G. Li, J. Hao, J. Hu, J. Wang, H. Simmons, S. Miura, Y. Mishina and G. Q. Zhao (2004). "BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways." Proc Natl Acad Sci U S A **101**(16): 6027-32.

Qin, B. Y., B. M. Chacko, S. S. Lam, M. P. de Caestecker, J. J. Correia and K. Lin (2001). "Structural basis of Smad1 activation by receptor kinase phosphorylation." Mol Cell **8**(6): 1303-12.

Randle, D. H., F. Zindy, C. J. Sherr and M. F. Roussel (2001). "Differential effects of p19(Arf) and p16(Ink4a) loss on senescence of murine bone marrow-derived preB cells and macrophages." Proc Natl Acad Sci U S A **98**(17): 9654-9.

Renshaw, M. W., M. A. Capozza and J. Y. Wang (1988). "Differential expression of type-specific c-abl mRNAs in mouse tissues and cell lines." Mol Cell Biol **8**(10): 4547-51.

Rheinwald, J. G., W. C. Hahn, M. R. Ramsey, J. Y. Wu, Z. Guo, H. Tsao, M. De Luca, C. Catricala and K. M. O'Toole (2002). "A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status." Mol Cell Biol **22**(14): 5157-72.

Rodda, S. J. and A. P. McMahon (2006). "Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors." Development **133**(16): 3231-44.

Rowley, J. D. (1973). "Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining." Nature **243**(5405): 290-3.

Russell, R. G., B. Espina and P. Hulley (2006). "Bone biology and the pathogenesis of osteoporosis." Curr Opin Rheumatol **18 Suppl 1**: S3-10.

Ruzinova, M. B. and R. Benezra (2003). "Id proteins in development, cell cycle and cancer." Trends Cell Biol **13**(8): 410-8.

Ruzinova, M. B., R. A. Schoer, W. Gerald, J. E. Egan, P. P. Pandolfi, S. Rafii, K. Manova, V. Mittal and R. Benezra (2003). "Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors." Cancer Cell **4**(4): 277-89.

Sakurai, H., H. Miyoshi, J. Mizukami and T. Sugita (2000). "Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase kinase by TAB1." FEBS Lett **474**(2-3): 141-5.

Sammar, M., S. Stricker, G. C. Schwabe, C. Sieber, A. Hartung, M. Hanke, I. Oishi, J. Pohl, Y. Minami, W. Sebald, S. Mundlos and P. Knaus (2004). "Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2." Genes Cells **9**(12): 1227-38.

Satow, R., A. Kurisaki, T. C. Chan, T. S. Hamazaki and M. Asashima (2006). "Dullard promotes degradation and dephosphorylation of BMP receptors and is required for neural induction." Dev Cell **11**(6): 763-74.

Satyanarayana, A. and K. L. Rudolph (2004). "p16 and ARF: activation of teenage proteins in old age." J Clin Invest **114**(9): 1237-40.

Schwartzberg, P. L., A. M. Stall, J. D. Hardin, K. S. Bowdish, T. Humaran, S. Boast, M. L. Harbison, E. J. Robertson and S. P. Goff (1991). "Mice homozygous for the ablm1 mutation show poor viability and depletion of selected B and T cell populations." Cell **65**(7): 1165-75.

Serrano, M., G. J. Hannon and D. Beach (1993). "A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4." Nature **366**(6456): 704-7.

Serrano, M., A. W. Lin, M. E. McCurrach, D. Beach and S. W. Lowe (1997). "Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a." Cell **88**(5): 593-602.

Shaw, F. H., D. E. Promislow, M. Tatar, K. A. Hughes and C. J. Geyer (1999). "Toward reconciling inferences concerning genetic variation in senescence in Drosophila melanogaster." Genetics **152**(2): 553-66.

Shay, J. W., O. M. Pereira-Smith and W. E. Wright (1991). "A role for both RB and p53 in the regulation of human cellular senescence." Exp Cell Res **196**(1): 33-9.

Shay, J. W. and I. B. Roninson (2004). "Hallmarks of senescence in carcinogenesis and cancer therapy." Oncogene **23**(16): 2919-33.

Shelton, D. N., E. Chang, P. S. Whittier, D. Choi and W. D. Funk (1999). "Microarray analysis of replicative senescence." Curr Biol **9**(17): 939-45.

Sherr, C. J. and J. D. Weber (2000). "The ARF/p53 pathway." Curr Opin Genet Dev **10**(1): 94-9.

Shi, Y. and J. Massague (2003). "Mechanisms of TGF-beta signaling from cell membrane to the nucleus." Cell **113**(6): 685-700.

Shibuya, H., H. Iwata, N. Masuyama, Y. Gotoh, K. Yamaguchi, K. Irie, K. Matsumoto, E. Nishida and N. Ueno (1998). "Role of TAK1 and TAB1 in BMP signaling in early Xenopus development." EMBO J **17**(4): 1019-28.

Shim, J. H., C. Xiao, A. E. Paschal, S. T. Bailey, P. Rao, M. S. Hayden, K. Y. Lee, C. Bussey, M. Steckel, N. Tanaka, G. Yamada, S. Akira, K. Matsumoto and S. Ghosh (2005). "TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo." Genes Dev **19**(22): 2668-81.

Sikder, H. A., M. K. Devlin, S. Dunlap, B. Ryu and R. M. Alani (2003). "Id proteins in cell growth and tumorigenesis." Cancer Cell **3**(6): 525-30.

Sini, P., A. Cannas, A. J. Koleske, P. P. Di Fiore and G. Scita (2004). "Abl-dependent tyrosine phosphorylation of Sos-1 mediates growth-factor-induced Rac activation." Nat Cell Biol **6**(3): 268-74.

Steelman, L. S., S. C. Pohnert, J. G. Shelton, R. A. Franklin, F. E. Bertrand and J. A. McCubrey (2004). "JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis." Leukemia **18**(2): 189-218.

Stevaux, O. and N. J. Dyson (2002). "A revised picture of the E2F transcriptional network and RB function." Curr Opin Cell Biol **14**(6): 684-91.

Suda, T., K. Miyama, et al. (1994). "Osteoporotic bone changes in SAMP6 mice are due to a decrease in osteoblast progenitor cells. In: Takeda, T., editor. The SAM model of senescence. Amsterdam Excerpta Medica: 47-52

Sun, L. Q., D. W. Lee, Q. Zhang, W. Xiao, E. H. Raabe, A. Meeker, D. Miao, D. L. Huso and R. J. Arceci (2004). "Growth retardation and premature aging phenotypes in mice with disruption of the SNF2-like gene, PASG." Genes Dev **18**(9): 1035-46.

Suzuki, A., R. S. Thies, N. Yamaji, J. J. Song, J. M. Wozney, K. Murakami and N. Ueno (1994). "A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo." Proc Natl Acad Sci U S A **91**(22): 10255-9.

Taagepera, S., D. McDonald, J. E. Loeb, L. L. Whitaker, A. K. McElroy, J. Y. Wang and T. J. Hope (1998). "Nuclear-cytoplasmic shuttling of C-ABL tyrosine kinase." Proc Natl Acad Sci U S A **95**(13): 7457-62.

Takada, K., M. Inaba, N. Ichioka, Y. Ueda, M. Taira, S. Baba, T. Mizokami, X. Wang, H. Hisha, H. Iida and S. Ikehara (2006). "Treatment of senile osteoporosis in SAMP6 mice by intra-bone marrow injection of allogeneic bone marrow cells." Stem Cells **24**(2): 399-405.

Takeda, T. (1999). "Senescence-accelerated mouse (SAM): a biogerontological resource in aging research." Neurobiol Aging **20**(2): 105-10.

Tang, J., G. M. Gordon, B. J. Nickoloff and K. E. Foreman (2002). "The helix-loop-helix protein id-1 delays onset of replicative senescence in human endothelial cells." Lab Invest **82**(8): 1073-9.

ten Dijke, P., O. Korchynskyi, G. Valdimarsdottir and M. J. Goumans (2003). "Controlling cell fate by bone morphogenetic protein receptors." Mol Cell Endocrinol **211**(1-2): 105-13.

Thiefes, A., S. Wolter, J. F. Mushinski, E. Hoffmann, O. Dittrich-Breiholz, N. Graue, A. Dorrie, H. Schneider, D. Wirth, B. Luckow, K. Resch and M. Kracht (2005). "Simultaneous blockade of NFkappaB, JNK, and p38 MAPK by a kinase-inactive mutant of the protein kinase TAK1 sensitizes cells to apoptosis and affects a distinct spectrum of tumor necrosis factor [corrected] target genes." J Biol Chem **280**(30): 27728-41.

Tournay, O. and R. Benezra (1996). "Transcription of the dominant-negative helix-loop-helix protein Id1 is regulated by a protein complex containing the immediate-early response gene Egr-1." Mol Cell Biol **16**(5): 2418-30.

Tsuji, K., A. Bandyopadhyay, B. D. Harfe, K. Cox, S. Kakar, L. Gerstenfeld, T. Einhorn, C. J. Tabin and V. Rosen (2006). "BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing." Nat Genet **38**(12): 1424-9.

Tybulewicz, V. L., C. E. Crawford, P. K. Jackson, R. T. Bronson and R. C. Mulligan (1991). "Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene." Cell **65**(7): 1153-63.

Urist, M. R. (1965). "Bone: formation by autoinduction." Science **150**(698): 893-9.

Van Etten, R. A. (1999). "Cycling, stressed-out and nervous: cellular functions of c-Abl." Trends Cell Biol **9**(5): 179-86.

Van Etten, R. A., P. Jackson and D. Baltimore (1989). "The mouse type IV c-abl gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization." Cell **58**(4): 669-78.

Varga, A. C. and J. L. Wrana (2005). "The disparate role of BMP in stem cell biology." Oncogene **24**(37): 5713-21.

Visnjic, D., Z. Kalajzic, D. W. Rowe, V. Katavic, J. Lorenzo and H. L. Aguila (2004). "Hematopoiesis is severely altered in mice with an induced osteoblast deficiency." Blood **103**(9): 3258-64.

von Bubnoff, A. and K. W. Cho (2001). "Intracellular BMP signaling regulation in vertebrates: pathway or network?" Dev Biol **239**(1): 1-14.

von Zglinicki, T. (2002). "Oxidative stress shortens telomeres." Trends Biochem Sci **27**(7): 339-44.

Wagner, K., P. Zhang, F. Rosenbauer, B. Drescher, S. Kobayashi, H. S. Radomska, J. L. Kutok, D. G. Gilliland, J. Krauter and D. G. Tenen (2006). "Absence of the transcription factor CCAAT enhancer binding protein alpha results in loss of myeloid identity in bcr/abl-induced malignancy." Proc Natl Acad Sci U S A **103**(16): 6338-43.

Wahl, G. M. and A. M. Carr (2001). "The evolution of diverse biological responses to DNA damage: insights from yeast and p53." Nat Cell Biol **3**(12): E277-86.

Waite, K. A. and C. Eng (2003). "From developmental disorder to heritable cancer: it's all in the BMP/TGF-beta family." Nat Rev Genet **4**(10): 763-73.

Wan, M. and X. Cao (2005). "BMP signaling in skeletal development." Biochem Biophys Res Commun **328**(3): 651-7.

Wang, E. (1995). "Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved." Cancer Res **55**(11): 2284-92.

Wang, S., M. C. Wilkes, E. B. Leof and R. Hirschberg (2005). "Imatinib mesylate blocks a non-Smad TGF-beta pathway and reduces renal fibrogenesis in vivo." FASEB J **19**(1): 1-11.

Wang, W., J. L. Martindale, X. Yang, F. J. Chrest and M. Gorospe (2005). "Increased stability of the p16 mRNA with replicative senescence." EMBO Rep **6**(2): 158-64.

Wang, Y., R. Benezra and D. A. Sassoon (1992). "Id expression during mouse development: a role in morphogenesis." Dev Dyn **194**(3): 222-30.

Weebadda, W. K., T. J. Jackson and A. W. Lin (2005). "Expression of p16INK4A variants in senescent human fibroblasts independent of protein phosphorylation." J Cell Biochem **94**(6): 1135-47.

Wei, S. and J. M. Sedivy (1999). "Expression of catalytically active telomerase does not prevent premature senescence caused by overexpression of oncogenic Ha-Ras in normal human fibroblasts." Cancer Res **59**(7): 1539-43.

Wen, S. T., P. K. Jackson and R. A. Van Etten (1996). "The cytostatic function of c-Abl is controlled by multiple nuclear localization signals and requires the p53 and Rb tumor suppressor gene products." EMBO J **15**(7): 1583-95.

Wieser, R., L. Attisano, J. L. Wrana and J. Massague (1993). "Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region." Mol Cell Biol **13**(12): 7239-47.

Wilkes, M. C. and E. B. Leof (2006). "Transforming growth factor beta activation of c-Abl is independent of receptor internalization and regulated by phosphatidylinositol 3-kinase and PAK2 in mesenchymal cultures." J Biol Chem **281**(38): 27846-54.

Winnier, G., M. Blessing, P. A. Labosky and B. L. Hogan (1995). "Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse." Genes Dev **9**(17): 2105-16.

Wong, S. and O. N. Witte (2004). "The BCR-ABL story: bench to bedside and back." Annu Rev Immunol **22**: 247-306.

Wong, W. K., J. A. Knowles and J. H. Morse (2005). "Bone morphogenetic protein receptor type II C-terminus interacts with c-Src: implication for a role in pulmonary arterial hypertension." Am J Respir Cell Mol Biol **33**(5): 438-46.

Wozney, J. M. (1998). "The bone morphogenetic protein family: multifunctional cellular regulators in the embryo and adult." Eur J Oral Sci **106 Suppl 1**: 160-6.

Wright, W. E. and J. W. Shay (2001). "Cellular senescence as a tumor-protection mechanism: the essential role of counting." Curr Opin Genet Dev **11**(1): 98-103.

Wu, C. H., J. van Riggelen, A. Yetil, A. C. Fan, P. Bachireddy and D. W. Felsher (2007). "Cellular senescence is an important mechanism of tumor regression upon c-Myc inactivation." Proc Natl Acad Sci U S A **104**(32): 13028-33.

Wu, X. B., Y. Li, A. Schneider, W. Yu, G. Rajendren, J. Iqbal, M. Yamamoto, M. Alam, L. J. Brunet, H. C. Blair, M. Zaidi and E. Abe (2003). "Impaired osteoblastic differentiation, reduced bone formation, and severe osteoporosis in noggin-overexpressing mice." J Clin Invest **112**(6): 924-34.

Xu, L. and J. Massague (2004). "Nucleocytoplasmic shuttling of signal transducers." Nat Rev Mol Cell Biol **5**(3): 209-19.

Xu, T., P. Bianco, L. W. Fisher, G. Longenecker, E. Smith, S. Goldstein, J. Bonadio, A. Boskey, A. M. Heegaard, B. Sommer, K. Satomura, P. Dominguez, C. Zhao, A. B. Kulkarni, P. G. Robey and M. F. Young (1998). "Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice." Nat Genet **20**(1): 78-82.

Yakar, S., C. J. Rosen, W. G. Beamer, C. L. Ackert-Bicknell, Y. Wu, J. L. Liu, G. T. Ooi, J. Setser, J. Frystyk, Y. R. Boisclair and D. LeRoith (2002). "Circulating levels of IGF-1 directly regulate bone growth and density." J Clin Invest **110**(6): 771-81.

Yamaguchi, K., S. Nagai, J. Ninomiya-Tsuji, M. Nishita, K. Tamai, K. Irie, N. Ueno, E. Nishida, H. Shibuya and K. Matsumoto (1999). "XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway." EMBO J **18**(1): 179-87.

Yamaguchi, K., K. Shirakabe, H. Shibuya, K. Irie, I. Oishi, N. Ueno, T. Taniguchi, E. Nishida and K. Matsumoto (1995). "Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction." Science **270**(5244): 2008-11.

Yanagita, M. (2005). "BMP antagonists: their roles in development and involvement in pathophysiology." Cytokine Growth Factor Rev **16**(3): 309-17.

Yi, S. E., A. Daluiski, R. Pederson, V. Rosen and K. M. Lyons (2000). "The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb." Development **127**(3): 621-30.

Ying, Q. L., J. Nichols, I. Chambers and A. Smith (2003). "BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3." Cell **115**(3): 281-92.

Yokota, Y. and S. Mori (2002). "Role of Id family proteins in growth control." J Cell Physiol **190**(1): 21-8.

Yoon, B. S., D. A. Ovchinnikov, I. Yoshii, Y. Mishina, R. R. Behringer and K. M. Lyons (2005). "Bmpr1a and Bmpr1b have overlapping functions and are essential for chondrogenesis in vivo." Proc Natl Acad Sci U S A **102**(14): 5062-7.

Yoon, B. S., R. Pogue, D. A. Ovchinnikov, I. Yoshii, Y. Mishina, R. R. Behringer and K. M. Lyons (2006). "BMPs regulate multiple aspects of growth-plate chondrogenesis through opposing actions on FGF pathways." Development **133**(23): 4667-78.

Yoshida, Y., S. Tanaka, H. Umemori, O. Minowa, M. Usui, N. Ikematsu, E. Hosoda, T. Imamura, J. Kuno, T. Yamashita, K. Miyazono, M. Noda, T. Noda and T. Yamamoto (2000). "Negative regulation of BMP/Smad signaling by Tob in osteoblasts." Cell **103**(7): 1085-97.

Yuan, Z.M., H. Shioya, T. Ishiko, X. Sun, J. Gu, Y.Y. Huang, H. Lu, S. Kharbanda, R. Weichselbaum and D.Kufe (1999). " p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage." Nature **399** (6738) : 814-7.

Zebedee, Z. and E. Hara (2001). "Id proteins in cell cycle control and cellular senescence." Oncogene **20**(58): 8317-25.

Zhang, H. and A. Bradley (1996). "Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development." Development **122**(10): 2977-86.
Zhang, J. and L. Li (2005). "BMP signaling and stem cell regulation." Dev Biol **284**(1): 1-11.

Zhang, J., C. Niu, L. Ye, H. Huang, X. He, W. G. Tong, J. Ross, J. Haug, T. Johnson, J. Q. Feng, S. Harris, L. M. Wiedemann, Y. Mishina and L. Li (2003). "Identification of the haematopoietic stem cell niche and control of the niche size." Nature **425**(6960): 836-41.

Zhao, M., S. E. Harris, D. Horn, Z. Geng, R. Nishimura, G. R. Mundy and D. Chen (2002). "Bone morphogenetic protein receptor signaling is necessary for normal murine postnatal bone formation." J Cell Biol **157**(6): 1049-60.

Zheng, W., H. Wang, L. Xue, Z. Zhang and T. Tong (2004). "Regulation of cellular senescence and p16(INK4a) expression by Id1 and E47 proteins in human diploid fibroblast." J Biol Chem **279**(30): 31524-32.

Zhu, H., P. Kavsak, S. Abdollah, J. L. Wrana and G. H. Thomsen (1999). "A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation." Nature **400**(6745): 687-93.

APPENDICES

APPENDIX A: BUFFERS FOR WESTERN BLOT AND IP ANALYSIS

BUFFER		Final concentration/vol
CO-IP buffer		
	Tris solution [pH7.4]	20mM
	NaCl	100mM
	NP-40	0.5%
	EDTA	0.5mM
	PMSF	0.5mM
	Protease inhibitor cocktail	0.5%

TBST Buffer (30X stock)

	Sodium Chloride	480g
	Tris base	152.4g

Top up to 2L using ddH_20 and adjust pH to 7.6 with 5M HCl. Dilute stock solution to 1X and add Tween 20 at 1/1000 (1ml per 1L solution), stir thoroughly before use.

APPENDIX B: CELL CULTURE REAGENT

PlatE culture medium

DMEM	-
FBS	10%
Pen/Strep	1X
Blasticidin	10ug/ml
Puromycin	1ug/ml

Prepared and filter-sterilized media is kept for not more than 1-2 months at 4^{o}C.

APPENDIX C: STAINING SOLUTIONS

Beta-galactosidase solution

X-Gal	0.1% (w/v)
Citric acid/ sodium phosphate (pH6.0)	40mM
Potassium ferrocyanide	5mM
Potassium ferricyanide	5mM
NaCl	150mM
MgCl	2mM

APPENDIX D: SDS-PAGE GEL COMPONENTS

Stacking gel (4%)

30% acrylamide (29:1)	1.3ml
ddH_2O	6.1ml
0.5M Tris-HCL pH 6.8	2.5ml
10% SDS	100ul
10% APS (w/v)	100ul
TEMED	10ul
Total volume	**10ml**

Resolving gel (15%)

30% acrylamide (29:1)	5.0ml
ddH_2O	2.36ml
1.5M Tris-HCL pH 8.8	2.5ml
10% SDS	100ul
10% APS (w/v)	100ul
TEMED	10ul
Total volume	**10ml**

APPENDIX E: PREPARATION OF DRUGS

Imatinib (STI571) (Novartis)

Dilute amphophilic pellet to 1mM stock concentration with sterile ddH2O. Dissolve thoroughly and store in aliquots at -20^{o}C. Stock can be directly used and diluted with media to achieve final concentration of 1uM (1000x dilution).

rhBMP2 (R&D systems and iDNA)

1mg lyphophilic pellet is diluted to 10ug/ml working stock with 1ml of dilution buffer (0.1% BSA in PBS). Dissolve thoroughly and store in aliquots at -20^{o}C. The same dilution buffer is used to further dilute BMP if necessary.

Mouse recombinant Noggin (R&D systems)

25ug lyophilized pellet dissolved with 500ul filtered PBS (with 0.1% BSA as carrier) to create a 50ug/ml working stock.

Mouse recombinant Chordin (R&D systems)

50ug lyophilized pellet dissolved with 500ul filtered PBS (with 0.1% BSA as carrier) to create a 100ug/ml working stock.

MEK inhibitor U0126 (Promega)

1mg pellet was resuspended with 234ul DMSO to prepare a 10mM working stock. Solution remains stable for 1 week at -20^{o}C.

Puromycin dihydrochloride (USBiological)

100mg of puromycin powder was weighed out and dissolved with 4mls of ddH_20 to prepare a working stock of 25mg/ml. Solution was filter-sterilized before storing in aliquots at -20^{o}C.