University of Alberta

Insights into the Role of Choline Kinase in Endochondral Bone Formation and Human Osteoblasts Function

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biochemistry

Spring 2014
Edmonton, Alberta

Abstract

Choline kinase (CK) phosphorylates choline to phosphocholine (PCho) and is the first enzyme in the CDP-choline pathway that generates phosphatidylcholine (PC). CK has three isoforms (CKα1, CKα2 and CKβ) that are encoded by two genes, *Chkα* and *Chkb*. Inactivation of *Chkα* results in embryonic lethality, whereas *Chkb*$^{-/-}$ mice display neonatal forelimb bone deformations. To understand the mechanisms underlying the bone deformations, we compared the biology and biochemistry of bone formation from embryonic to neonatal *Chkb*$^{-/-}$ and *Chkb*$^{+/+}$ mice. The deformations are specific to the radius and ulna and occur during the late embryonic stage. The radius and ulna of *Chkb*$^{-/-}$ mice display expanded hypertrophic zones, unorganized proliferative columns in their growth plates, and delayed formation of primary ossification centers. The differentiation of chondrocytes in the radius and ulna of *Chkb*$^{-/-}$ mice was impaired, as was chondrocyte proliferation and expression of matrix metalloproteinases 9 and 13. In chondrocytes from *Chkb*$^{-/-}$ mice, PC was slightly lower than in wild-type mice whereas the amount of PCho was decreased by approximately 75%. In addition, the radius and ulna from *Chkb*$^{-/-}$ mice contained fewer osteoclasts along the cartilage/bone interface. These data indicate that CKβ plays an important role in endochondral bone formation by modulating growth plate physiology.

There is limited knowledge about PC biosynthesis in bone formation. Thus, we characterized PC metabolism in both primary human osteoblasts (HOB) and human osteosarcoma MG-63 cells. Our results show that the CDP-choline pathway is the only *de novo* route for PC biosynthesis in both HOB and MG-63

cells. Both CK activity and CKα expression in MG-63 cells were significantly higher than in HOB cells. Silencing of CKα in MG-63 cells had no significant effect on PC concentration but decreased the amount of PCho by approximately 80%. Silencing of CKα also reduced cell proliferation. Moreover, pharmacological inhibition of CK activity impaired the mineralization capacity of MG-63 cells. These data suggest that CK and its product PCho are required for the normal growth and mineralization of MG-63 cells.

In summary, these studies outline the physiological importance of CKβ in murine endochondral bone formation and CKα in human osteoblasts function.

Acknowledgements

First and foremost, I thank my supervisor Dr. Dennis Vance for offering me the opportunity and resources to work on these interesting and challenging projects. It has been a rewarding and memorable experience.

I thank Dr. Jean Vance for her great suggestions on the manuscripts.

I thank my supervisory and examining committee members for the helpful advice and comments throughout my graduate program and review of this work.

I thank Dr. Gengshu Wu, the past member in Vance lab, who initiated the two projects in my thesis.

Special thanks to Dr. Frank Beier at the University of Western Ontario. I am appreciative for the training opportunity as a visiting student in his lab to learn various experimental techniques in bone biology and the valuable insights he provided to my study.

I thank Susanne Lingrell and Randy Nelson for excellent technical assistance.

I would like to thank all the past and present members of the Vance lab and the MCBL.

Lastly, I like to acknowledge my family. I am very grateful for the support from my parents and the constant love from my wife. Thank you all for the encouragement throughout many years of school in Canada.

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Abbreviations

ACH	achondroplasia
AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
ADP	adenosine diphosphate
ALP	alkaline phosphatase
apo	apolipoprotein
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
CCD	cleidocranial dysplasia
cDNA	complementary deoxyribonucleic acid
CDP	cytidine diphosphate
CE	cholesteryl ester
CK	choline kinase
CL	cardiolipin
CM	chylomicrons
CMP	cytidine monophosphate
CPT	CDP-choline: 1, 2-diacylglycerol cholinephosphotransferase
CT	CTP:phosphocholine cytidylyltransferase
CTP	cytidine triphosphate

DAB	diaminobenzidine
DG	diacylglycerol
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDF	growth and differentiation factor
GH	growth hormone
GST	glutathione *S*-transferase
GTP	guanosine triphosphate
h	hour
HC-3	hemicholinium-3
HDL	high density lipoprotein
HF/HC	high fat/high cholesterol
HIF-1α	hypoxia-inducible factor-1α
HMG	high mobility group

HOB	primary human osteoblasts
IGF-1	insulin-like growth factor 1
IGF1R	insulin-like growth factor 1 receptor
Ihh	Indian hedgehog
IU	international unit
K_m	Michaelis constant
LC-MS	liquid chromatography-mass spectrometry
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LPC	lysophosphatidylcholine
LRP1	low density lipoprotein receptor related protein 1
MAPK	mitogen-activated protein kinase
M-CPT1b	muscular carnitine-palmitoyl transferase 1b
M-CSF	macrophage colony stimulating factor
MDR2	multiple-drug resistant protein 2
Micro-CT	micro computed tomography
min	minute
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid

NMR	nuclear magnetic resonance
nSMase2	neutral sphingomyelinase 2
OPG	osteoprotegerin
Osx	osterix
PC	phosphatidylcholine
PCho	phosphocholine
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEA	phosphoethanolamine
PEMT	phosphatidylethanolamine *N*-methyltransferase
PI	phosphatidylinositol
Pi	inorganic phosphate
PI3K	phosphatidylinositol 3-kinase
PPi	pyrophosphate
PPR	PTH/PTHrP receptor
PS	phosphatidylserine
Ptch1	patched-1
PTH	parathyroid hormone

PTHrP	parathyroid hormone-related protein
Ral-GDS	Ral guanine nucleotide dissociation stimulator
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
RNA	ribonucleic acid
RNAi	RNA interference
RPM	revolutions per minute
Runx2	runt-related transcription factor 2
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Shox	short stature homeobox
shRNA	short hairpin RNA
siRNA	small interfering RNA
SM	sphingomyelin
Smo	smoothened
TG	triglyceride
TRAP	tartrate-resistant acid phosphatase
µm	micrometre
µCi	microcurie

VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein

Chapter 1

Introduction

1.1 Phosphatidylcholine

Phosphatidylcholine (PC) was originally described in 1847 as a constituent of egg yolk and was named ‘lecithin’ after *lekithos*, the Greek equivalent for egg yolk (1). A few years later, it was demonstrated that lecithin has two fatty acids chains esterified to a glycerol backbone, and a choline head group attached to the third hydroxyl group through a phosphodiester linkage (2) (Figure 1.1). Over 20 different PC species have been identified in mammalian cells and the diversity of PC molecules is based on the variety of fatty acids varying in length and the number and position of double bonds. Often the sn-1 position is occupied by saturated fatty acids (e.g., 16:0 palmitic acid), while polyunsaturated fatty acids are usually found in sn-2 position (e.g., 20:4, arachidonic acid) (2).

1.2 Biological Roles of Phosphatidylcholine

1.2.1 Phosphatidylcholine as a component of cellular membranes

PC is the most abundant phospholipid within mammalian membranes, usually accounting for more than 50% of membrane phospholipids, and more than 30% of total cellular lipid mass (1, 3). Due to the generally cylindrical shape and amphipathic nature, PC spontaneously organizes into bilayers (4), thus it is ideally served as the structural elements of biological membranes. The unsaturated acyl chains in PC are kinked and therefore allow the fluidity on the membrane. In addition, PC content influences physiological properties of the membrane including fluidity, integrity and curvature (5, 6). The distribution of PC also varies among different cell types and organelles (5), and proper PC distribution is important for cellular and organelle functions (7).

1.2.2 Phosphatidylcholine as a component of bile

The components of bile include bile acids, cholesterol and phospholipids. Bile is produced in the liver and functions in the intestine to assist lipid digestion and dietary fat absorption (8). The liver secretes a significant portion of PC across

the apical membrane into bile, representing ~90% of total bile lipid (9). In mice, the daily amount of PC secreted into bile is almost equivalent to the total amount of PC in the liver (10, 11). The transport of PC into bile is facilitated by multiple-drug resistant protein 2 (MDR2), a PC-specific flippase that actively translocates PC across the hepatic membrane into bile (12). Depletion of *mdr2* gene in mice results in liver disease due to impaired PC secretion into bile, demonstrating the importance of biliary PC secretion (12).

1.2.3 Phosphatidylcholine as a component of lung surfactant

Pulmonary surfactant consists of mixtures of lipids and proteins secreted by alveolar type II epithelial cells (13). Pulmonary surfactant is critical for lung function by reducing the surface tension at the air-water interface and preventing alveolar collapse (13). A specific PC species, dipalmitoylphosphatidylcholine, accounts for ~80% of the total lipid content (13). The essential role of PC for pulmonary surfactant is demonstrated in mice in which disruption of the major pathway for PC biosynthesis in alveolar type II epithelial cells results in neonatal respiratory failure because of reduced levels of dipalmitoylphosphatidylcholine (14).

1.2.4 Phosphatidylcholine as a component of lipoproteins

Lipoproteins transport hydrophobic lipids throughout the body via the circulatory system. Structurally, all lipoproteins consist of a monolayer of phospholipids, cholesterol and apolipoproteins, which form a spherical shell surrounding the hydrophobic core of triacylglycerols (TG) and cholesteryl esters (15). The different types of lipoproteins are classified based on their sizes and densities including chylomicrons (CM), very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins. CM particles have the largest size but lower density due to the TG-rich core, whereas HDL particles are smallest in diameter but with greater density because of carrying a higher proportion of proteins (15). Each lipoprotein class carries featured apolipoprotein components.

For instance, apolipoprotein B is characteristic of VLDL and LDL particles and apolipoprotein A-1 is characteristic of HDL particles (15).

Generally, the total phospholipid content of lipoproteins increases with density and is also directly proportional to the surface area. PC by far is the predominant phospholipid component of all lipoproteins, with levels ranging from 60-80% (16). Other phospholipid components include sphingomyelin, lyso-PC, phosphatidylethanolamine (PE), phosphatidylserine and phosphatidylinositol (16).

1.2.5 Phosphatidylcholine biosynthesis

In mammalian cells PC is synthesized via two different pathways (17). The major pathway for PC biosynthesis is the cytidine diphosphate (CDP)-choline pathway, also called Kennedy pathway (18, 19) (Figure 1.2). The CDP-choline pathway utilizes choline as initial substrates to produce PC and consists of three enzymatic steps. In the first reaction, choline kinase phosphorylates choline to phosphocholine (PCho) using adenosine triphosphate (ATP). In the following reaction, CTP: phosphocholine cytidylyltransferase (CT) converts phosphocholine to CDP-choline with cytidine triphosphate (CTP). In the last reaction, CDP-choline: 1, 2-diacylglycerol cholinephosphotransferase (CPT) catalyzes the formation of PC via the exchange of cytidine monophosphate (CMP) for diacylglycerol.

The second pathway for PC biosynthesis occurs by three sequential methylations of PE by phosphatidylethanolamine *N*-methyltransferase (PEMT) (17) (Figure 1.2). The PEMT pathway is only quantitatively significant in liver where it accounts for approximately 30% of total hepatic PC production and the remaining 70% is produced via the CDP-choline pathway (20).

1.3 Phosphatidylcholine Biosynthesis: The CDP-choline Pathway

1.3.1 Choline

The majority of choline is acquired from the diet as free choline or esterified choline (21). Choline can be also *de novo* synthesized through the methylation of PE to PC in PEMT pathway followed by catabolism of PC hydrolyzed by phospholipases to release the choline moiety (22). Choline carries a positive charge; therefore, active transport is required for choline to cross the plasma membrane (23). Three choline transport systems have been identified: (a) 'facilitated diffusion' described in erythrocytes driven by choline concentration gradient (24); (b) 'high affinity, sodium dependent transport' often coupled to neurotransmitter acetylcholine production in neuronal cells (25), (c) 'low affinity, sodium independent transport' found in most cells to import choline for phospholipids synthesis (26).

The primary fate of choline is the substrate to synthesize PC via the CDP-choline pathway (22). Excess choline can undergo irreversible oxidation in the kidney and liver to generate betaine (27), which is utilized as a methyl donor in the homocysteine-methionine methylation pathway (28).

The nutritional importance of choline was first demonstrated in 1932 by Charles Best (29). It is now recommended that choline is an essential dietary requirement for humans, and the recommended Adequate Intake (AI) is 550 mg/day and 425 mg/day for men and women respectively (21). Individuals at risk for choline deficiency particularly are pregnant and lactating women and infants (30). Choline deficiency can induce hepatic fat accumulation, which leads to liver steatosis and dysfunction (31, 32). In addition, choline is a precursor for acetylcholine synthesis (33), and choline deficiency could impair normal brain development because of impaired acetylcholine generation (34). Furthermore, choline may play a role in musculoskeletal development, as choline deficiency has been linked to skeletal muscle damages (35, 36).

1.3.2 Choline kinase

Choline kinase (CK) is a cytosolic enzyme that phosphorylates choline to yield phosphocholine (PCho) in the presence of ATP and magnesium (37). Although CK catalyzes the first reaction in the CDP-choline pathway for PC biosynthesis, CK is not considered as the key regulatory enzyme. Another enzyme in the pathway, CTP: phosphocholine cytidylyltransferase (CT) usually catalyzes the rate-limiting reaction (38, 39). CK activity was first demonstrated in 1953 by Wittenberg and Kornberg in Brewer's yeast (37). The enzyme was then purified from rat kidney and was also shown to have ethanolamine kinase activity (40-42). In mammalian cells including humans, CK exists in three isoforms (CKα1, CKα2 and CKβ) that are encoded by two separate genes: *Chkα* located on chromosome 19 and *Chkb* located on chromosome 15 (43, 44). While *Chkb* only encodes a single protein CKβ (395 amino acids), alternative splicing of *Chkα* results in two variants: CKα1 (439 amino acids) and CKα2 (457 amino acids), which differ only by the presence of an additional 18-residue insert starting at position 155 in CKα2 (44). CKβ is approximately 60% identical in sequence to CKα1 and CKα2 (44). In addition, both in mice and humans, the CKβ protein is produced by a *bicistronic* gene which also generates the muscular carnitine-palmitoyl transferase 1b (M-CPT1b) protein (45). Because of the overlapping between CKβ and M-CPT1b, it is likely that the regulatory element that controls one gene could affect the expression of the other (46).

Both CKα and CKβ are ubiquitously expressed, although CKα is abundantly expressed in testis and liver and high expression of CKβ is found in liver and heart (44). Active CK requires the formation of either homo- or hetero-dimers, and neither isoform is active in the monomeric form (44). In mouse liver, ~60% of the total CK activity was contributed by α/β heterodimers and α/α or β/β homodimers represent the remaining (43). In addition, recent studies have identified several essential domains and amino-acid residues important for the formation of active CKα and CKβ dimers in human and mouse by structural and mutational analysis (47, 48).

The choline kinase and ethanolamine kinase activity of both CKα and CKβ have been previously described in different mammalian cells (44). A recent study further characterized the enzymatic properties of both CK isoforms in vitro (49). Michaelis constant (K_m) measures the kinetics of an enzyme and inversely correlates with the affinity of the enzyme for substrates (50). In this study, the *in vitro* kinase activity assay demonstrated that CKβ has a higher K_m for choline than CKα, but CKα has a higher K_m for ethanolamine than CKβ. These results suggest that in cell-free system choline is a better substrate for CKα than CKβ, whereas ethanolamine is a better substrate for CKβ than CKα.

1.3.3 CTP: phosphocholine cytidylyltransferase

CTP: phosphocholine cytidylyltransferase (CT) catalyzes the conversion of phosphocholine to CDP-choline, which is considered as the rate-limiting step in CDP-choline pathway to produce PC (38, 39). In mammalian cells, CT exists as three isoforms, encoded by two separate genes: *Pcyt1a* and *Pcyt1b* (51, 52). The predominant isoform in most tissues is CTα encoded by *Pcyt1a*, located on chromosome 16 (51, 52). *Pcyt1b* is located on the X chromosome and encodes two isoforms: CTβ1 and CTβ2 in humans, and CTβ2 and CTβ3 in mice (51, 52). CTβ isoforms have low expression in most tissues but are highly expressed in brains and reproductive organs (52).

CTα is largely localized to the nucleus of many cell types due to the presence of an N-terminal nuclear localization signal (53). CTα also has a core catalytic domain, a membrane-binding domain and a phosphorylation domain (54). CTβ isoforms are localized in the cytoplasm and lack the nuclear localization signal but have similar domain structure as CTα (51-54).

CT forms homodimers in cells and is primarily regulated by the reversible translocation from a cytosolic, inactive form to a membrane-bound form that is active (55). The association to the membrane is believed to activate CT by relieving an inhibitory restraint in the catalytic domain (17). In addition, it has

been shown that several lipid ligands, including diacylglycerol (56), fatty acid (57), oxysterols (58) and low PC levels (59), are able to stimulate the translocation and activation of CT. In contrast, high PC levels (60) prevent the association of CTα to the membrane for a negative feedback regulation of CT activity and PC biosynthesis. Therefore, the regulatory mechanism allows for a rapid modulation of PC biosynthesis in response to the availability of lipid precursors and alterations in membrane composition (17).

The physiological function of each CT isoform has been revealed by generation of CT-deficient mice. Global depletion of *Pcyt1a* in mice resulted in early embryonic lethality following fertilization (day 3.5) (61). Therefore, the Cre-lox system was utilized to disrupt CT isoforms in specific tissues. Targeted deletion of CTα in lung epithelial cells led to severe respiratory failure due to a significant decrease of the major pulmonary surfactant phospholipid, dipalmitoylphosphatidylcholine (14). In addition, depletion of hepatic CTα altered the lipoprotein homeostasis and resulted in reduced plasma level of HDL and VLDL because of impaired secretion (62, 63). Hepatic CTα-deficient mice also develop liver steatosis (hepatic fat accumulation), which is believed to be caused by reduced VLDL secretion due to decreased supply of hepatic PC (64).

CTβ2-deficient mice survive but exhibit gonadal dysfunction (65). These animals have reduced fertility due to either lack of ovarian follicles or impaired spermatogenesis. Although CTβ2 is highly expressed in the brain, CTβ2-deficient mice have no obvious neurological defects. However, it has been reported that CTβ2 plays an important role in regulating neuronal outgrowth and axon branching (66, 67).

1.3.4 CDP-choline: 1, 2-diacylglycerol cholinephosphotransferase

The final step of the CDP-choline pathway is catalyzed by CDP-choline: 1, 2-diacylglycerol cholinephosphotransferase (CPT) (17), which was first discovered by Kennedy and co-workers (18, 19). The cell fractionation study has

shown that the majority of CPT is located on the ER but it is also found on the Golgi and nuclear membrane (68). CPT is an integral membrane-bound protein, so the protein purification of this enzyme has been extremely difficult and it has never been successfully purified (17). However, two human cDNAs (CHPT1 and CEPT1) have been cloned and expressed based on the sequence homology to the yeast CPT cDNA (69, 70). CHPT1 specifically uses CDP-choline as the substrate, whereas CEPT1 utilizes both CDP-choline and CDP-ethanolamine (69, 70). In most studies, CPT activity has been found in excess in the cells, suggesting that the amount of CPT does not limit PC biosynthesis (17). However, it appears that the CPT reaction is regulated by the supply of its two substrates: CDP-choline and diacylglycerol (71).

1.4 Phosphatidylcholine Biosynthesis: The Phosphatidylethanolame *N*-Methyltransferase Pathway

1.4.1 Characterization of phosphatidylethanolame *N*-methyltransferase

Phosphatidylethanolame *N*-methyltransferase (PEMT) catalyzes the conversion of PE to PC via three sequential methylation reactions (17) (Figure 1.2). PEMT pathway is the second major pathway to synthesize PC, but only quantitatively significant in liver, producing ~30% of hepatic PC with the remaining 70% contributed by CDP-choline pathway (20). The *Pemt* gene is located on chromosome 11 in mice and chromosome 17 in humans (72). PEMT is localized to the ER and mitochondria-associated-membrane (a subfraction of the ER) (73), and has four transmembrane domains (74). Although PEMT is an intrinsic membrane protein, it was successfully purified from rat liver microsomes (75).

1.4.2 Phosphatidylethanolame *N*-methyltransferase and liver failure

To understand the physiological importance of PEMT, its knockout mice (*Pemt*$^{-/-}$ mice) have been generated. However, when fed on chow diet, *Pemt*$^{-/-}$

mice have normal hepatic levels of PC and PE, hepatocyte morphology and plasma lipid profile (76). To further understand the physiological function of PEMT pathway, *Pemt*$^{-/-}$ mice were fed a choline-deficient diet to limit the availability of choline for the CDP-choline pathway (77). These animals rapidly develop steatosis, steatohepatitis and severe liver failure within 3 days. Analysis of choline-deficient livers of *Pemt*$^{-/-}$ mice revealed a ~50% decrease in hepatic PC content, which was initially thought to be the reason for liver failure. However, it was demonstrated later that decreased PC/PE ratio was responsible for the liver phenotypes in *Pemt*$^{-/-}$ mice (78). This study led to a proposed model that PC molecules that are cylindrically shaped, in *Pemt*$^{-/-}$ mice hepatocyte cell surface, are replaced by PE molecules that are inverted cone-shaped, resulting in lower PC/PE ratio (78). The possible consequence of the PC/PE replacement and lower PC/PE ratio is that the plasma membrane bilayer has improper packing and thus becomes permeable, leading to membrane leakage that promotes inflammation associated with steatohepatitis and eventually liver failure (78).

1.4.3 Phosphatidylethanolame *N*-methyltransferase, atherosclerosis and obesity

Atherosclerosis is the major cause of cardiovascular disease, and is characterized by the artery wall thickening as a result of the accumulation of lipids (79, 80). Several risk factors are associated with atherosclerosis including elevated plasma homocysteine (81, 82) and increased plasma level of VLDL and LDL (79, 80).

In the reaction catalyzed by PEMT, three molecules of *S*-adenosylhomocysteine are formed (Figure 1.2), and subsequently hydrolyzed to homocysteine (17, 83). In *Pemt*$^{-/-}$ mice, the plasma level of homocysteine is significantly decreased (84, 85). Furthermore, in liver-specific CTα-deficient mice, PEMT activity is increased by two-fold that results in elevated plasma homocysteine concentration (86). Thus, inhibition of PEMT activity may be

protective against cardiovascular disease partially by reducing plasma homocysteine levels.

Elevated circulating level of VLDL and LDL are known risk factors for atherosclerosis (79, 80). Although *Pemt*$^{-/-}$ mice fed on chow diet appear normal (76), when challenged with high fat/high cholesterol (HF/HC) diet, *Pemt*$^{-/-}$ mice have ~50% less plasma triglyceride (TG) and apolipoprotein (apo) B100, indicating impaired VLDL secretion (85). In addition, hepatocytes isolated from *Pemt*$^{-/-}$ mice displayed a significant decrease in secreted apoB100 and TG compared with hepatocytes from wild type littermates (87). Thus, Vance and co-workers proposed that the hypolipidemic effect of PEMT may mediate cardioprotective effects (84). This hypothesis was confirmed by knockout of *Pemt* in two well-established mouse models of atherosclerosis: the LDL receptor-deficient mouse (*Ldlr*$^{-/-}$) and the apoE-null (*ApoE*$^{-/-}$) mouse, both spontaneously develop atherosclerosis on HF/HC or chow diet, respectively (88, 89). *Pemt*$^{-/-}$*Ldlr*$^{-/-}$ mice fed on HF/HC diet showed an 80% reduction in atherosclerotic lesion area and a significant reduction in plasma TG and cholesteryl ester (CE) (84). Furthermore, these animals also have decreased hepatic VLDL secretion and increased VLDL clearance from plasma. Similar to *Pemt*$^{-/-}$*Ldlr*$^{-/-}$ mice, *Pemt*$^{-/-}$ *ApoE*$^{-/-}$ mice also display an improved atherogenic plasma lipoprotein profile (45% decrease in TG and 25% decrease in CE), reduced atherosclerotic lesion area (30%) as well as reduced cardiac TG content attenuating cardiac dysfunction (90).

PEMT is also linked to obesity as *Pemt*$^{-/-}$ mice fed on high fat diet are protected against obesity compared to wild type littermates (91). Despite having fatty liver, *Pemt*$^{-/-}$ mice gained less body weight, had increased energy expenditure and maintained normal insulin sensitivity when compared to wild type littermates. Interestingly, when additional choline was supplemented to the diet, *Pemt*$^{-/-}$ mice gained weight, had normal energy expenditure and developed insulin resistance, suggesting that the beneficial physiological effects in *Pemt*$^{-/-}$ mice were due to insufficient choline rather than impaired PC biosynthesis.

1.5 Physiological Functions of Choline Kinase

1.5.1 Regulation of choline kinase

Choline kinase (CK) acts as a dimeric enzyme and the proportion and different combination of homo or heterodimer populations have been proposed to be tissue-specific (43). The α/α homodimers have been proposed to be the most active form, β/β homodimers are the less active form, and the α/β heterodimers have an intermediate phenotype (43).

In addition to the regulation of CK enzymatic activity by different homo- or hetero-dimerization, CK is also regulated by alternative mechanisms including transcriptional regulation. Early studies have shown that aromatic polycyclic hydrocarbon can stimulate CK enzymatic activity in rat liver (92, 93), which can be blocked by the treatment of protein biosynthesis inhibitors, cycloheximide or actinomycin, suggesting that the elevated enzymatic activity could be due to the change in the enzyme level (94). In addition, CKα, but not CKβ enzymatic activity can be induced in mouse liver by carbon tetrachloride, CCl_4 (43, 95). The induction of CK enzymatic activity by CCl_4 was shown to be caused by increased binding of the transcription factor c-jun to Activating Protein-1 (AP-1) element found in CKα distal promoter, resulting in increased gene expression (95). Studies of the 5' flanking sequence of murine *Chkα* also revealed more responsive elements in the distal promoter including XRE, CCAAT and CREB (44, 96). Similar study was also performed for the 5'flanking region of murine *Chkb*, but no distal promoter with acute responsive elements has been found (44). In addition, human *Chkα* has been identified as a gene target for hypoxia-inducible factor-1α (HIF-1α) in a human cancer model, and binding of H1F-1α to the hypoxia response elements within the promoter induced *Chkα* expression (97). Moreover, several reports have demonstrated regulation of CK activity by the actions of hormones and various growth factors such as epidermal growth factor, insulin, prolactin and vasopressin (98). Small GTPases are hydrolase enzymes that bind and hydrolyze guanosine triphosphate (GTP) and generally serve as

molecular switches for a number of cellular signalling events (99). It has been shown that two small GTPases, Ras and RhoA, are upstream activators of *Chkα* expression (100-102). However, no GTPase has been shown to activate *Chkb* expression through protein-protein interaction (102). Thus, more research is required for the characterization of transcriptional regulation of *Chkb*.

1.5.2 CKα and embryogenesis

To evaluate the physiological function of each CK isoform, mice lacking either CKα or CKβ have been created or identified. *Chkα*$^{-/-}$ mice were generated with an embryonic stem cell line that has an insertional mutation in *Chkα* gene. *Chkα*$^{-/-}$ mice have early embryonic lethality around day 3.5 and CKα activity accounts for ~80% of total CK activity in mouse embryonic fibroblasts (103), suggesting that CKα plays an indispensable role for mouse early embryogenesis. In growing mouse embryos, cells require more PC to meet the need of rapid dividing and proliferation, thus, PC biosynthesis may be impaired due to dramatically decreased CK activity in *Chkα*$^{-/-}$ mice, which could explain the early embryonic lethality (104). However, heterozygous mutant *Chkα*$^{+/-}$ mice appear normal during their embryonic development and have no abnormality in gross anatomy (103). Furthermore, although ~30% CK activity reduction resulted in choline accumulation and decreased phosphocholine (PCho) in liver and testis of *Chka*$^{+/-}$ mice, the amount of PC was not altered when compared to wild type littermates (103). These results suggest that adult mice do not need full CKα expression to maintain normal PC level in liver and testis, supporting the traditional assumption that CK is not usually the rate-limiting enzyme in the CDP-choline pathway to produce PC.

1.5.3 CKβ and muscular dystrophy

A spontaneous genomic deletion in murine *Chkb* leads to muscular dystrophy and neonatal forelimb bone deformation (105). *Chkb*$^{-/-}$ mice have rostral-to-caudal gradient of severity with very mild forelimb but severe hindlimb

muscular dystrophy. However, *Chkb*$^{-/-}$ mice have no obvious abnormality in other organs such as brain, skin and heart. In the following study, impaired PC biosynthesis, choline accumulation and decreased PCho in hindlimb muscles of *Chkb*$^{-/-}$ mice were observed (106). Phospholipase A_2 and phospholipase C hydrolyze PC and respectively generate lysoPC and fatty acid, and diacylglycerol and PCho (107). Both activities of PC-phospholipase A2 and phospholipase C were increased in hindlimb muscle of *Chkb*$^{-/-}$ mice, indicating enhanced PC turnover (106). Furthermore, hindlimb muscle in *Chkb*$^{-/-}$ mice has morphological changes in mitochondrial structure and distribution, that is, enlarged mitochondria at the periphery of muscle fibers and absence of mitochondria in the center of muscle fibers, suggesting compromised mitochondrial function (105, 106). In addition, injecting mice with CDP-choline, which can bypass the reaction catalyzed by CK, increased PC content and improved the muscle damage phenotype (106). Thus, the authors concluded that the hindlimb muscular dystrophy was contributed by decreased PC biosynthesis, enhanced PC catabolism and impaired mitochondrial function (106).

A subsequent study revealed some mechanisms behind the very mild phenotype in the forelimb muscle (108). Despite ~50% decrease of CK activity, the forelimb muscle of *Chkb*$^{-/-}$ mice maintains a stable PC homeostasis and has a significant CK activity compensation from CKα. Furthermore, it was found that CKα is the dominant isoform in forelimb muscle whereas CKβ is the major isoform in hindlimb muscle. Therefore, the muscular dystrophy is not significantly developed in forelimb but predominantly manifested in hindlimb as a result of *Chkb* loss.

Recessive mutations in *Chkb* causing the congenial muscular dystrophy in humans have also been identified. In 1998, four Japanese patients from three unrelated families were reported to have congenital muscular dystrophy (109). These patients displayed perinatal hypotonia and experienced a delay in motor development. Furthermore, the muscle sections from the patients exhibited mitochondrial structural abnormality similar to what was observed in *Chkb*$^{-/-}$ mice:

giant mitochondria at the periphery of muscle fibers and mitochondrial depletion in the center of the muscle fibers. In addition, ten Turkish and one British patients were newly identified to have very similar muscle pathological phenotypes to the Japanese patients (110). All the patients were screened for mutations, and homozygous or compound heterozygous mutations of *Chkb* were identified (111). These mutations were predicated to truncate either the essential consensus sequences or the catalytic domains of CK. Indeed, CKβ expression was absent and CK activity was undetectable in the biopsied muscles from these patients (111), indicating that the muscular dystrophy is caused by the loss-of-function mutations in *Chkb*.

In *Chkb*$^{-/-}$ mice, despite significant CK activity reduction, PC mass was only decreased in hindlimb muscle but maintained stable in organs other than muscle including liver, brain, kidney and heart (108), suggesting that the lack of *Chkb* on PC homeostasis is tissue-specific. In human patients, PC content is only decreased by approximately 25% in muscle despite the loss of CK activity (111), also suggesting that a little bit of CK activity could be sufficient for PC biosynthesis.

1.5.4 Choline kinase and bone development

In addition to the hindlimb muscular dystrophy phenotype in *Chkb*$^{-/-}$ mice, another striking phenotype is neonatal forelimb bone deformation (105). Unlike muscular dystrophy, no CKβ mutations in humans have been associated with bone disease. Furthermore, no previous study has demonstrated the direct connection between CK and skeletal development. However, deficiency of choline, the substrate of CK, is linked to abnormal bone development in turkeys and impaired bone remodeling in rats (112-114). In addition, the products of the reactions catalysed by CK, PCho and phosphoethanolamine (PEA) are implicated in the function of a recently identified enzyme, phosphatase orphan 1, or Phospho1 (115).

Phospho1 is a member of the haloacid dehalogenase superfamily, which is a large group of magnesium-dependent hydrolases (115). Phospho1 was first identified in the chick, and it is expressed approximately 100-fold higher in mineralizing than non-mineralizing tissues (116). Furthermore, immunolocalization studies have shown that Phospho1 is specifically localized to mineralizing regions of skeletal tissue (117). Since its identification in chick, Phospho1 orthologues have also been found in a number of other species, including humans, mice and zebra fish (118, 119). Human and murine Phospho1 are ~94% identical to each other and both are ~62% identical to chick Phospho1 (117). In addition, Phospho1 is ~42% identical to Phospho2, another member of the haloacid dehalogenase family that, however, is currently not implicated in bone mineralization (115). Phospho1 is a soluble cytosolic enzyme that has high phosphohydrolase activity toward two phospholipid metabolites, PCho and PEA, to generate inorganic phosphate (Pi) (120, 121). It has been shown that PCho and PEA are the two most abundant phospho-monoesters in cartilage (122). However, Phospho1 does not hydrolyze a number of other phosphate-containing molecules such as adenosine triphosphate (ATP), adenosine diphosphate (ADP) and pyrophosphate (PPi) (120). Several studies have demonstrated the importance of Phospho1 for the mineralization process (123-125). Inhibition of Phospho1 activity by small molecule inhibitors significantly decreased the mineralization capacity in vitro (123). Furthermore, pharmacological inhibition of Phospho1 activity led to impaired skeletal mineralization during limb development in the chick (124). In addition, *Phospho1* knockout mice have decreased bone mineral density and deformed limb bones (125).

PCho and PEA acting as substrates for Phospho1 to generate Pi raises the possibility that CK may play a role in skeletal mineralization, as most intracellular PCho is produced by CK (19, 38), which also has ethanolamine kinase activity to generate PEA (40-42). Much less PCho accumulation was found in neonatal rat calvaria compared to liver tissue (126), which could be explained by the speculation that up-regulated Phospho1 expression stimulated enhanced PCho

hydrolysis. PCho and PEA are respectively involved in PC and phosphatidylethanolamine (PE) synthesis, and conversely, PC and PE can be hydrolyzed by phospholipase C to generate PCho and PEA, respectively (127). In addition, the proportion of membrane phospholipids containing these two groups decreases during mineralization with accumulation of 1, 2-diacylglycerol, indicative of phospholipase C activity (128). Therefore, a novel mechanism has been proposed whereby Pi locked within the plasma membranes may be released from PCho and PEA via the action of Phospho1 and phospholipase C to generate the Pi concentration required for normal mineralization (115, 129).

Interestingly, another enzyme in the CDP-choline pathway, CT, has also been recently linked to skeletal development (130, 131). Two very recent human genetic studies showed that homozygous mutations in *Pcyt1a*, which encodes CT, were identified in patients with spondylometaphyseal dysplasias (130, 131), a rare autosomal-recessive disorder characterized by short stature, progressive lower-limb bowing, flattened vertebrae, metaphyseal abnormalities and visual impairment caused by cone-rod dystrophy (132-134). Since CT is the regulatory enzyme in the CDP-choline pathway to synthesize PC (38, 39), the authors proposed that loss-of-function mutations of CT could cause attenuated PC biosynthesis during the skeletal development of those patients, which eventually leads to the pathological phenotypes.

1.6 Choline Kinase in Cell Transformation and Tumorigenesis

1.6.1 Choline kinase and its product phosphocholine are new biomarkers for cancer

Both choline kinase (CK) and its reaction product phosphocholine (PCho) have been involved in cell proliferation, transformation and tumorigenesis (98, 135-137). High levels of CK expression and activity and elevated levels of PCho are frequently observed in cell lines derived from human tumors as well as in different tumor tissues (98, 135-137). For example, increased CKα expression

was found in epithelial ovarian cancer (138) and in bladder carcinomas (139). Furthermore, higher CK activity and increased levels of PCho were detected in human colon cancer (140) and in 1, 2-dimethylhydrazine-induced rat colon cancer (141), as well as in human breast carcinomas (142). In addition, CKα expression is significantly increased in a large panel of tumor-derived cell lines and in lung, prostate and colorectal cancers (143). Moreover, a clinical study has shown that patients with non-small cell lung cancer, whose tumors exhibit overexpression of CKα, display a statistically significant poor diagnosis (144). The increased CKα expression in various cancer cells is often mediated by Ras and RhoA proteins from small GTPase family, through a signalling pathway that involves two of the best known effectors: Ral guanine nucleotide dissociation stimulator (Ral-GDS) and phosphatidylinositol 3-kinase (PI3K) (100-102).

A link between CKα and cell proliferation has also been established. Increased DNA synthesis in response to insulin-like growth factor 1(IGF-1) and insulin was observed in murine fibroblasts (145), and overexpression of CKα in human primary mammary epithelial cells stimulated the entry into S phase (DNA synthesis phase) of the cell cycle (146). In addition, a recent microarray study has shown that CKα overexpression induces the transcriptional up-regulation of genes involved in cell proliferation and promotes cell cycle progression (147).

Production of PCho has also been described as an essential process in cell proliferation induced by growth factors in different types of human cells (148-150). Inhibition of CK activity in these cells decreased the intracellular PCho levels and further led to a blockage of DNA synthesis. Moreover, elevated PCho levels are common characteristic in cell lines derived from human tumors (151-152). Cell transformation involves an alteration of phospholipid metabolism, which accompanies an increase of intracellular PCho concentration (136, 148, 153). In addition, studies using nuclear magnetic resonance (NMR) techniques have shown increased levels of PCho in a variety of murine and human tumors (154-156). These findings indicate that higher levels of PCho are associated with

cell malignancy and seen as a biomarker useful for monitoring the cancer progression.

Most studies on CK in cancer research are focused on the CKα isoform. The involvement of CKβ in cell transformation and carcinogenesis has also been recently studied. It was shown that, both in vitro and in vivo, CKβ was not able to induce tumor growth in the condition where CKα does (49). In addition, gene expression profile of CK isoforms in various cancer cell lines showed that CKα is significantly overexpressed but not CKβ (49, 138, 152). These findings imply that CKα plays a more important role than CKβ in the carcinogenic process.

1.6.2 Pharmacological inhibition and RNA interference mediated targeting of choline kinase

Because of the relevance of CKα in tumorigenesis, CKα-targeted inhibition has been proposed as an efficient anti-cancer strategy. Many small-molecule compounds have been synthesized and tested as CKα inhibitors, and most of them are derivatives of hemicholinium-3 (HC-3), a compound with structural similarity to choline (157). These compounds exhibit anti-proliferation activity *in vitro* and anti-tumor activity *in vivo* (135, 158, 159). X-ray crystallographic studies on CK isoforms in complex with HC-3 also showed that HC-3 is a more potent inhibitor of CKα than of CKβ (160). More recently, MN58b has been synthesized and identified as the most effective inhibitory compound for CKα, due to its more specific and profound inhibitory effect against CKα under *in vivo* conditions (139, 146, 158).

Although multiple CK inhibitors display efficient antitumor activity, the toxicity and side effects associated with pharmacological inhibition are a major concern. As an alternative approach, RNA interference (RNAi) offers a new strategy to selectively down-regulate the expression of CKα in mammalian cells. The specific ablation of CKα expression induced apoptosis in tumor cells without affecting the normal cells (161). Furthermore, knockdown of CKα with plasmid-

based short hairpin RNA (shRNA) reduced proliferation but stimulated the differentiation in breast cancer cells (162). In addition, lentivirus-mediated down-regulation of CKα expression reduced the growth of a human breast cancer xenograft (163). Also, combinational treatments with CKα knockdown using RNAi and anticancer agent 5'-fluorouracil significantly enhanced the antitumor effect in breast cancer cells, compared with 5'-fluorouracil treatment alone (164).

Moreover, it has been recently demonstrated that selective inhibition of CK via RNAi or inhibitory compounds markedly impairs Akt activation by reducing Akt phosphorylation at Ser473 in the PI3K/Akt pathway (165), which plays an important role in regulating many cellular processes including growth, metabolism and survival (166). Xenograft tumors treated with CKα inhibitors exhibited a significant reduction in Akt (Ser473) phosphorylation, which correlated well with a regression of these tumors in the mouse model (165). Thus, CKα plays an essential role in regulating Akt (Ser474) phosphorylation, thereby enhancing cell survival and proliferation. Also, another two recent studies showed that selective inhibition of CKα using RNAi or small-molecule inhibitors simultaneously supressed both mitogen-activated protein kinase (MAPK) and PI3K/Akt signalling, indicating that CK activity is required for amplification of signalling cascades needed for cancer cell survival and growth (167, 168). Therefore, these studies imply a novel mechanism in which CK is involved in carcinogenesis.

1.7 Endochondral Bone Formation

1.7.1 Overview

Bone is a form of highly specialized mineralized connective tissue that provides strength to the skeleton. Two distinctive types of osseous tissues exist in bone: cortical bone and trabecular bone (169). Cortical bone, or compact bone, forms a very dense and hardened shell around the bone surface, providing the strength and protection; trabecular bone, or cancellous bone, in contrast, is mainly

present at the ends of the bones, and is rigid, vascular but spongy, with a higher surface area. Bone can be also classified into five categories based on the shape: long bone, short bone, flat bone, irregular bone and sesamoid bone (169). Long bones are longer than they are wide, and mainly located in the limbs (169). The anatomy of murine limb long bone mimics that of human, in which proximal humerus and distal radius/ulna are located in the forelimb, and proximal femur and distal fibula/tibia are located in the hindlimb (169) (Figure 1.3).

Bone formation occurs through two different mechanisms: some skeletal elements including craniofacial bones are formed by a process of intramembranous ossification, whereby bone tissues are formed directly from the differentiation of mesenchymal cells; most skeletal elements including limb long bones are formed by endochondral ossification that involves the remodeling of initial cartilaginous template into bone tissues (169). Endochondral bone formation is a multi-stage process that starts with the commitment of mesenchymal cells to a chondrogenic cell lineage (170-173) (Figure 1.4). These cells aggregate into condensations at the sites of future skeletal elements. In the limbs, these condensations initiate proximally and then extend distally. Cells in these mesenchymal condensations differentiate into chondrocytes and produce the cartilage extracellular matrix (ECM) rich in type II collagen and proteoglycan aggrecan. In contrast to round resting chondrocytes that proliferate at very slow rate, the flat proliferating chondrocytes divide rapidly along the longitudinal axis of the bone and form characteristic parallel columns of cells. These cells then withdraw from the cell cycle, initiate terminal differentiation, and become prehypertrophic and then hypertrophic chondrocytes. These chondrocytes are characterized by changes in gene expression and a dramatic increase in cell volume. The mature hypertrophic chondrocytes express predominantly type X collagen and are able to mineralize the cartilage ECM and ultimately die by apoptosis, leaving the mineralized matrix as a scaffold for future bone formation.

In the cartilage elements during endochondral ossification, a thin layer of mesenchymal cells on the periphery of the condensations forms the

perichondrium, which is subsequently developed into the periosteum. Cells in perichondrium invade the area of hypertrophic cartilage, along with blood vessels, osteoblasts (bone-forming cells) and osteoclast (bone-resorbing cell) precursors. Collectively, these cells degrade and remodel the cartilage ECM, and osteoblasts deposit a bone-specific ECM in the bone center to form the primary ossification centre. At the two ends of the bones, secondary ossification centers are formed by a similar mechanism. The round resting, flat proliferating and enlarged prehypertrophic and hypertrophic chondrocyte cells caught between the primary and secondary ossification centers constitute the growth plate. Growth plate activity is responsible for lengthening the skeletal elements and controls longitudinal bone growth. Numerous disorders characterized by retarded growth have their origins in altered growth plate physiology (174).

1.7.2 The Sox Trio: a master regulator of early chondrogenesis

Sox proteins are key transcription factors that control chondrogenesis (170-173). Members of the Sox family of transcription factors contain a high mobility group (HMG)-box DNA binding domain, and are closely related to SRY, the male sex-determination transcription factor (175, 176). Twenty *Sox* genes have been identified in human and mouse genomes (175, 176), and three of them, *Sox9*, *Sox5* and *Sox6* have been shown to play an essential role in regulating chondrogenesis (170-173).

A number of studies have led to the conclusion that commitment of cells to chondrogenic mesenchymal condensation and to chondrogenic differentiation requires the transcription factor Sox9 (170-173). In humans, heterozygous mutations in *Sox9* cause campomelic dysplasia, a rare disorder of skeletal development that results in short stature and bowing of limb long bones (177, 178). In most cases, the disease leads to perinatal lethality due to respiratory complications that are mainly caused by poorly formed tracheal and rib cartilage (177, 178). During mouse embryogenesis, Sox9 is expressed in all chondroprogenitors and proliferative chondrocytes, but its expression is excluded

in hypertrophic chondrocytes (179-181). Sox9 stimulates the transcription of a number of cartilage matrix genes including *Col2a1* that encodes type II collagen and *aggrecan* that encodes proteoglycan aggrecan (181-183). The phenotypes in heterozygous Sox9 mutant mice (*Sox9*$^{+/-}$ mice) mimic the skeletal manifestations of campomelic dysplasia (184). In these *Sox9*$^{+/-}$ mice, cartilage hypoplasia is caused by smaller mesenchymal condensation, and the growth plates also display an enlargement of hypertrophic zone, suggesting that sufficient level of Sox9 is required for both proper formation of mesenchymal condensation and proper control of chondrocyte differentiation (184). The essential role of Sox9 in chondrogenesis was also demonstrated by studying the fate of Sox9-null cells in wild type chimeric mice (185). These chimeric mice were created by injecting Sox9-null embryonic stem cells, with *LacZ* knocked in as a reporter gene, into wild-type blastocysts. Analysis of these chimeras showed that Sox9-null cells could not participate in condensation and failed to differentiate into chondrocytes (185). In separate genetic experiments, when Cre recombinase-LoxP system was employed to selectively knock out *Sox9* in early limb mesenchyme, no cartilage condensation was formed and chondrogenic marker genes were not expressed, further supporting the requirement of Sox9 for the commitment of mesenchymal cells to a chondrogenic cell fate (186).

Two other members of the Sox family, Sox5 and Sox6, are also important transcription factors in chondrogenesis. Although both Sox5-null and Sox6-null mutant mice only show mild cartilage phenotypes, Sox5/Sox6 double mutant mice die *in utero* with severe defects in cartilage formation (187). However, in these double mutants, Sox9 expression is not altered and mesenchymal condensation forms normally. These observations suggest that Sox5 and Sox6 are required at the step that follows the formation of mesenchymal condensation during chondrogenesis. Genetic study of Sox9 mutants shows that, when Sox9 is inactivated in limb mesenchyme prior to mesenchymal condensation formation, expression of Sox5 and Sox6 are abolished (186), suggesting that Sox9 is also required for the activation of Sox5 and Sox6 in chondrocytes.

1.7.3 Regulation of chondrocyte proliferation and differentiation by Ihh/PTHrP pathway

Indian hedgehog (Ihh), a mammalian homolog of the Drosophila Hedgehog secreted ligand, plays a critical role in coordinating chondrocyte proliferation and differentiation (188). During endochondral bone formation, Ihh is expressed by prehypertrophic and early hypertrophic chondrocytes and binds to its receptor Patched-1 (Ptch1), a multipass membrane protein (188, 189). As a result, Ptch1 inhibition of a second membrane protein Smoothened (Smo) is lost, and the release of Smo repression triggers a cascade for Ihh signal transduction to take place (189). Ihh stimulates chondrocyte proliferation and inhibits chondrocyte hypertrophy (190-193). Ihh homozygous mutant ($Ihh^{-/-}$) mice have normal skeletal element formation but exhibit a severe growth defects as early as embryonic stage and display a marked decrease in chondrocyte proliferation (191). In addition, the hypertrophic chondrocytes in the growth plates of $Ihh^{-/-}$ mice extend to the ends of the bones, suggesting that the proliferating chondrocytes leave the proliferative pool prematurely (191). Consistent with this model, a cartilage-specific deletion of *Smo* also led to decreased chondrocyte proliferation, similar to what was observed in $Ihh^{-/-}$ mice (193). In addition, gain-of-function experiments in the same study showed that either cartilage-specific transgenic expression of Ihh or a constitutively active form of Smo promotes chondrocyte proliferation (193).

Parathyroid hormone-related protein (PTHrP) is named due to its resemblance to parathyroid hormone (PTH), the major regulator of blood calcium in birds and mammals (194). Unlike PTH, PTHrP is present only in very low amounts in the circulation and it is synthesized locally in a variety of tissues including growth plates (195). PTHrP acts on the same G-protein-coupled receptor used by PTH, PTH/PTHrP receptor (PPR), which is expressed at low levels in proliferating chondrocytes and at high levels by prehypertrophic/hypertrophic chondrocytes (196). In the absence of either PPR or PTHrP in mice, the proliferation zones are much shorter than normal and the

zones of hypertrophic chondrocytes are close to the ends of bones (190, 197, 198). In contrast, when PTHrP is overexpressed or active PPR is constitutively expressed in cartilage, the differentiation of chondrocytes into hypertrophic chondrocytes is delayed (199, 200). In addition, expression of constitutively active PPR in chondrocytes of *Ihh*$^{-/-}$ mice rescues the early hypertrophy phenotype (201). Thus, PTHrP acts to keep chondrocytes proliferating and delay the chondrocyte maturation, as well as Ihh expression. On the other hand, the expression of PTHrP is totally dependent on the expression of Ihh (191, 192, 198). Ihh is believed to act directly on PTHrP-producing cells, as removal of Smo from these cells prevents PTHrP expression (202).

The interaction between Ihh and PTHrP leads to a proposed model in which these two factors together regulate a negative feedback loop (170-173) (Figure 1.5). PTHrP is secreted from the cells at the top of the growth plate, then diffuses away from the site of production to act on its receptors in proliferating chondrocytes to keep them in a proliferative pool and delay the hypertrophic differentiation as well as Ihh expression. When chondrocytes escape from the control of PTHrP, they stop proliferation, undergo hypertrophy and synthesize Ihh. Then Ihh can stimulate the production of PTHrP and chondrocyte proliferation. Because PTHrP is synthesized at the ends of the bones, and Ihh is expressed only in prehypertrophic/hypertrophic cells, the interaction between PTHrP and Ihh leads to a distance separation of their synthesizing cells, thus limits the actions of each signalling system. Therefore, Ihh/PTHrP pathway has physiological importance in assuring the optimal coordination between chondrocyte proliferation and differentiation.

1.7.4 Runx2 and Runx3, the master inducers of chondrocyte hypertrophy

Both Runx2 (previously known as Cbfa1) and Runx3 are members of the small family of Runt-domain transcription factors (170-173). Runx2 was initially characterized as an essential transcription factor required for osteoblast differentiation and bone formation in both membranous and endochondral skeletal

elements (203-205). Runx2 is also expressed in cartilage, at very low levels in chondrogenic mesenchymal condensation, but its expression increases in prehypertrophic/hypertrophic chondrocytes (170-173). Runx2 promotes the full progression of hypertrophic differentiation of chondrocytes (170-173). In Runx2-deficient mouse embryo, there is complete absence of endochondral bones and the chondrocyte maturation is severely inhibited (206, 207). In addition, when Runx2 is ectopically expressed in immature chondrocytes, it induces the premature hypertrophy of chondrocytes by stimulating the expression of *Col10a1* that encodes type X collagen, and other hypertrophic markers, both *in vivo* (208, 209) and *in vitro* (210). Runx2 also activates *Ihh* promoter and therefore stimulates its expression (211). Furthermore, PTHrP can inhibit Runx2 expression (212), which may partially explain its ability to delay the chondrocyte hypertrophy. These observations suggest that Runx2 contributes to the Ihh/PTHrP negative feedback loop to maintain the proper balance between chondrocyte proliferation and maturation.

The transcription factor Runx3 also plays an important role in promoting chondrocyte hypertrophy in cooperation with Runx2 (211). Runx3-deficient mouse embryos have a delay in endochondral bone formation, and deletion of both *Runx2* and *Runx3* led to complete absence of prehypertrophic/hypertrophic chondrocytes (211). Therefore, Runx2 has a dual role: it is needed for osteoblast differentiation and, together with Runx3, is also required for chondrocyte hypertrophy.

1.7.5 Regulation of cartilage matrix degradation by matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of extracellular matrix (ECM)-degrading enzymes that share common functional domains and activation mechanisms (213). These enzymes are synthesized as a secreted or transmembrane proform and then processed to a mature and active form by the removal of an amino-terminal propeptide (214). MMPs are extracellular

regulators of cell growth, migration and ECM remodeling and they are produced by a variety of cells including epithelial cells, fibroblasts and inflammatory cells (215). It has been known that several MMPs are involved in skeletal development, including MMP2, MMP7, MMP8, MMP9, MMP13 and MMP14 (214).

During endochondral bone formation, the cartilage intermediate is ultimately replaced by bone tissues, a process that requires the extensive degradation of cartilage ECM surrounding the hypertrophic chondrocytes (213). The cartilage ECM degradation is also a prerequisite for the subsequent vascular invasion of the hypertrophic cartilage by ossification front including blood vessels, osteoblasts and osteoclasts (173). Particularly two MMPs, MMP9 and MMP13, have been identified as the main proteolytic enzymes responsible for these degradation events. MMP9 appears to be expressed in osteoclast precursors, rather than the chondrocytes (216). MMP9-deficient mice before weaning have significant shortening of the long bones, in which the growth plates also reveal a profound lengthening of the hypertrophic zone (216). Furthermore, MMP9-deficient mice also show decreased trabecular bone formation, vascular invasion, osteoclast recruitment and delayed primary ossification (216). However, a full recovery of the phenotypes was observed after about 4 weeks of age. These results indicate that MMP9 acts to favour cartilage removal and promote vascular invasion.

In contrast to MMP9, MMP13 is mainly expressed in hypertrophic chondrocytes (217). Similar growth plate phenotypes to that of MMP9-deficient mice were observed in MMP13-deficient mice: chondrocytes undergo normal hypertrophy, but the hypertrophic zone is significantly expanded and the invasion of the ossification front is delayed (218, 219). Interestingly, deletion of both *Mmp9* and *Mmp13* genes in mice results in more dramatically expanded hypertrophic zones and a more substantial delay in primary ossification than deletion of either gene alone (219). These findings suggest that MMP9 and MMP13 are synergistic in their effects on the regulation of cartilage ECM degradation during endochondral ossification.

1.7.6 Vascular invasion of growth cartilage by the ossification front

The degradation of cartilage ECM by MMPs facilitates the invasion of hypertrophic cartilage by the ossification front, including blood vessels for angiogenesis, osteoclasts for cartilage ECM resorption and osteoblasts for matrix mineralization, which eventually leads to the replacement of cartilage by bone (173, 213). During this process, osteoclasts are required for cartilage matrix resorption and the subsequent establishment of the primary ossification center, as in their absence no vascular invasion or bone matrix deposition occurs (220). MMP9 is critical for osteoclast recruitment as MMP9-deficient mice exhibit a delay in osteoclast recruitment during vascular invasion (216, 221). In addition, an in vitro study also demonstrated that MMP9 is required for the migration of osteoclast precursors to the resorption site (222).

Angiogenesis factors are expressed in developing bones, and particularly, vascular endothelial growth factor (VEGF) expressed by the terminal hypertrophic chondrocytes, plays an essential role in the vascular invasion (173, 213). VEGF is produced in three isoforms, VEGF 120, VEGF 164 and VEGF 188, generated by mRNA alternative splicing (223). It is believed that the availability of VEGF is regulated by MMP9 activity. This hypothesis is based on the observations that a significant compensational increase of VEGF mRNA level in MMP9-deficient mice and a delayed angiogenic response induced by cartilage explants from MMP9-deficient mice (224). VEGF is a chemoattractant for osteoclasts, and invasion of osteoclasts into hypertrophic cartilage requires VEGF, since inhibition of VEGF activity blocks the osteoclast recruitment (221, 225). Furthermore, deletions of specific VEGF isoforms in mice result in expansion of hypertrophic zones and delayed vascular invasion and primary ossification (226-228), which phenocopy that of MMP9-deficient mice (216). Thus, a model has been proposed for the complementary interplay between MMP9 and VEGF during endochondral bone formation (173, 213). VEGF is present in cartilage and produced by hypertrophic chondrocytes. Upon degradation of cartilage ECM by

MMP9, the sequestered VEGF is released and acts to attract osteoclast precursors and to favor the vascular invasion and cartilage replacement by bone.

1.7.7 Fibroblast growth factor signalling in endochondral bone development

The fibroblast growth factor (FGF) family consists of 23 ligands that bind to FGF receptors, a family of four membrane-spanning tyrosine kinase receptors (FGFR1-4) (229). Ligand binding to the extracellular domain of FGFR leads to intracellular tyrosine kinase activation and often the triggering of mitogen activating protein kinase (MAPK)-based signalling cascade that results in gene activation (230).

FGFR2 is expressed in condensing mesenchyme during the early stage of endochondral bone development, suggesting that FGF signalling likely plays a role in initiating early cartilage development (231). Consistent with this finding, FGF signalling stimulates the expression of Sox9 (232), the key transcription factor regulating chondrogenesis (170-173).

The first functional evidence for the importance of FGF signalling in skeletal development actually came from the striking finding that gain-of-function mutations in FGFR3 result in two types of short-limbed dwarfism in human, a severe achondroplasia (ACH) (233, 234) and a less severe hypochondroplasia (235). Several animal models have demonstrated that FGFR3 negatively regulates chondrocyte proliferation (236-238). Activating mutations of *Fgfr3* in mice lead to dwarfism and ACH (236, 237), whereas depletion of *Fgfr3* in mice results in increased chondrocyte proliferation and enlarged skeletons (238). The ligand responsible for FGFR3 signalling appears to be FGF18, as *Fgf18* knockout mice exhibit enhanced skeletal growth that closely resembles that of FGFR3-deficient mice (239-241).

MAPK is a major downstream effector of FGF18-FGFR3 signalling pathway. A recent study showed that ectopic activation of MAPK signalling produces an ACH phenotype, similar to what was observed in activating FGFR3

mutants (242). In contrast, blocking MAPK activation rescues ACH phenotype obtained with constitutively active FGFR3 (243).

Moreover, it has been demonstrated that FGFR3 represses chondrocyte proliferation through activation of the transcription factor STAT1 (244-246), which mediates anti-proliferative effects and is linked to direct regulation of cell cycle inhibitors (247). In addition, knockout of *Fgfr3* gene in mice increases Indian hedgehog (Ihh) expression but activation of FGFR3 decreases Ihh expression (248, 249), suggesting that FGF signalling negatively regulates the expression of Ihh, which plays a critical role in promoting chondrocyte proliferation (190-193). Thus, these findings reveal another potential mechanism in which FGFR3 decreases chondrocyte proliferation by suppressing Ihh expression, in addition to the STAT1-based growth regulatory mechanism.

1.7.8 Bone morphogenetic proteins in endochondral bone development

Bone morphogenetic proteins (BMPs), also known as growth and differentiation factors (GDFs), are members of the large TGFβ superfamily of secreted signalling molecules (250). They were first discovered for their remarkable capability to induce cartilage and bone formation when injected subcutaneously in mice (170-173). BMPs bind to and activate cell surface type I and type II receptors that are serine-threonine protein kinases (251). Activation of these receptors induces the phosphorylation and activation of downstream effector SMAD proteins, which then move from the membrane to the nucleus where they act as transcription factors (252).

BMPs play a variety of essential roles in endochondral bone development, as early as during the formation of mesenchymal condensations and subsequent chondrogenesis (170-173). BMP antagonist Noggin blocks the formation of mesenchymal condensations and interferes with subsequent chondrogenesis (253). In contrast, primordial cartilage is enlarged in Noggin-deficient mice (254). In

addition, formation of chondrocytes in mice was suppressed by disruption of two BMP type I receptors (255).

After chondrocyte formation, BMPs also regulate chondrocyte proliferation and differentiation. BMP2 and BMP6 are expressed in pre-hypertrophic and hypertrophic chondrocytes and BMP7 is expressed in proliferating chondrocytes (256). Addition of BMPs to murine limb explants increases chondrocyte proliferation whereas Noggin represses chondrocyte proliferation (249, 257). These observations indicate that BMP signalling stimulates chondrocyte proliferation in growth cartilage. However, the effects of BMPs on chondrocyte differentiation have been variable, probably due to multiple actions of BMPs and different state of differentiation of the target chondrocytes (258, 259).

BMP signalling, particularly GDF5, also has a critical role on the joint formation. Joints between endochondral bones originate by segmentation of an initial continuous condensation followed by apoptosis occurring where the joints are formed for cavitation (260). Mice with mutations in GDF5 display normal cartilage condensation up to the time of segmentation, but then specific joints fail to form, resulting in fusion of phalanges, wrist and ankle bones, and certain other joints (261-263). These findings reveal the importance of GDF5 in stimulating the segmentation and inducing proper apoptosis. Consistent with the mice model, null GDF5 mutation has been identified in human acromesomelic chondrodysplastic diseases, and these skeletal disorders are characterized by short limbs, altered shape of bones and particularly missing phalangeal joints (264).

1.7.9 Growth hormone and insulin-like growth factor 1

Growth hormone (GH) is a peptide hormone that is secreted from pituitary gland, whereas insulin-like growth factor 1 (IGF-1), encoded by *igf-1* gene in mammals, is mainly produced in liver as an endocrine hormone (265). The primary action of GH and IGF-1 is mediated by binding to their specific receptors

on the cell surface, GH-receptor for GH and IGF-1 receptor (IGF1R) for IGF-1 (265). GH and IGF-1 have been known for many years as important stimulators of longitudinal bone growth (265). Children with excess GH due to pituitary adenomas suffer from gigantism (265). In contrast, impaired growth is seen in children with GH deficiency or insensitivity due to GH-receptor mutations (266-268). GH deficiency or insensitivity does not affect prenatal growth significantly (265). Conversely, IGF-1 deficiency due to mutations in *igf-1* gene or IGF-1 insensitivity as a result of mutations in *IGF1R* impairs both prenatal and postnatal growth (269, 270).

Consistent with the role in humans, the importance of GH/IGF-1 axis for longitudinal bone growth in mice has also been demonstrated by genetic targeting. Disruption of the gene encoding GH in mice results in normal birth weight, but impaired postnatal growth (271). Mice lacking *igf-1* gene or IGF-1 receptor show intrauterine growth retardation with dramatic birth weight reduction (272). In addition, mice deficient in liver-derived IGF-1 production have reduced circulating IGF-1 level and impaired longitudinal bone growth (273), which suggests that circulating IGF-1 is the critical source for maintenance of normal skeletal growth.

The effect of GH on skeletal growth is mediated by liver-derived IGF-1 (265). Administration of IGF-1 markedly improves linear bone growth in mutant mice and human patients with inactivating mutations in GH receptor (274, 275), supporting the important role of circulating IGF-1 for skeletal growth. Moreover, direct injection of GH into the growth plate accelerates the longitudinal bone growth, indicative of local action of GH on the growth plate (276). Some studies demonstrated that the local action of GH on the growth plate could be mediated by increased local production of IGF-1 that acts in a paracrine/autocrine fashion to stimulate chondrogenesis (277, 278). These findings suggest that local IGF-1, in addition to circulating IGF-1, is also an essential source for regulating longitudinal bone growth.

1.7.10 Calcium and phosphate homeostasis in bone mineralization

Calcium and phosphate ions are the main components of hydroxyapatite crystals in the mineralized bone tissues, and their levels in circulation play an essential role in mineralization. Defective regulation of calcium and phosphate causes disease of osteomalacia, characterized by hypomineralized skeleton (279, 280). Two important hormones are critical for maintaining calcium and phosphate levels in the body, vitamin D and parathyroid hormone (PTH).

Vitamin D is synthesized from previtamin D_3 or provided by daily diet (281, 282). Vitamin D_3 is synthesized through the action of sunlight exposure on its precursor 7-dehydrocholesterol (282). Vitamin D_3 is first hydroxylated in the liver at position 25 to make 25-hydroxyvitamin D_3 (25(OH) D_3), then transported to kidney and hydroxylated again to form 1, 25-dihydroxyvitamin D_3 (1, 25$(OH)_2$ D_3) (282), which is the active form and also the ligand for vitamin D receptor (VDR). Binding of 1, 25-dihydroxyvitamin D_3 to VDR induces the gene expression of several transport proteins involved in intestinal calcium absorption (283). In general, vitamin D maintains skeletal calcium and phosphate balance by stimulating calcium and phosphate absorption in the intestine and assisting the proper functioning of PTH to maintain blood calcium and phosphate levels (280). Thus, vitamin D deficiency leads to decreased bone mineral density and an increased risk of osteoporosis or bone fracture (283). In addition, the ablation of VDR in mice results in hypocalcaemia, osteomalacia and impaired bone formation (284, 285). Furthermore, vitamin D influences growth plate physiology, as mice lacking VDR have widening and thickening of the growth plates, decreased apoptosis of hypertrophic chondrocytes, and delayed vascular invasion and primary ossification (286, 287).

PTH is exclusively secreted by parathyroid gland and the secretion is stimulated by hypocalcaemia (194). PTH works through three mechanisms to increase calcium levels (288): (a) PTH stimulates the release of calcium and phosphate from the bone by up-regulating osteoclast activity to enhance bone

resorption. (b) PTH decreases the urine loss of calcium but increases the urine loss of phosphate. (c) PTH promotes the absorption of calcium and phosphate in the small intestine by stimulating the synthesis of 1, 25-dihydroxyvitamin D_3 in the kidney. The end result of PTH release is an increase of calcium but small net drop of phosphate in the circulation.

Matrix vesicles are small membrane-invested particles, released by budding from the surface of chondrocytes or osteoblasts (289). Matrix vesicles are the initial site of mineralization and have elevated level of alkaline phosphatase (ALP) (290), which is the main phosphatase to yield inorganic phosphate for bone mineralization (291-293). Humans and mice lacking functional ALP have decreased bone mineralization (293-295). Furthermore, ALP also involves the hydrolysis of pyrophosphate (296), which is a known inhibitor of hydroxyapatite crystal formation (297). The accumulation of pyrophosphate impairs skeletal mineralization, whereas extracellular pyrophosphate deficiency leads to enhanced bone formation in the skeleton (298, 299). Thus, pyrophosphate hydrolysis promotes the mineralization of matrix vesicles and the balance between local level of phosphate and pyrophosphate is critical for the control of mineralization (300).

1.8 Osteoblasts in Bone Formation and Osteoclasts in Bone Resorption

Bone is a dynamic tissue that undergoes constant adaption to maintain skeletal size, shape and structure integrity. Bone modelling is a process that is responsible for bone growth and adaption that require both formation and bone removal (resorption) (169). Two types of cells are involved in bone modelling, osteoblasts for bone formation and osteoclasts for bone resorption.

The bone matrix is produced by bone-forming cells osteoblasts, which are derived from bone marrow mesenchymal stem cells (301-303). Osteoblasts are differentiated from mesenchymal progenitor cells into proliferating pre-osteoblasts, then mature bone-matrix producing osteoblasts. The master activator

of osteoblast differentiation is Runx2 (runt-related transcription factor 2). In Runx2-deficient mice, osteoblasts fail to differentiate and the formation and mineralization of bone are completely absent (203-205). In addition, mice lacking only one allele of *Runx2* display hypoplastic clavicles and delayed closure of the fontanelles, a phenotype that mimics the human disease cleidocranial dysplasia (CCD) (304, 305). Genetic analysis of CCD patients revealed heterozygous mutations of *Runx2* gene, thus demonstrating the critical role of Runx2 in human osteoblast differentiation (304, 305). Osterix (Osx) is a zinc finger-containing transcription factor expressed in osteoblasts and is also an obligatory factor for normal osteoblast differentiation. Osx-deficient mice are perinatal lethal due to arrested osteoblast maturation, and lack mineralized bone, similar to Runx2-deficient mice but less severe (306). The finding that Runx2 is expressed in Osx-deficient mice but Osx is not expressed in Runx2-deficient mice is an indication that Osx acts downstream of Runx2 in the transcriptional cascades of osteoblast differentiation (306).

Various growth factors also modulate osteoblast proliferation, differentiation and maturation including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), parathyroid hormone (PTH) (302). The fully differentiated osteoblasts are characterized by expression of alkaline phosphatase and type I collagen, both of which are important for bone matrix production and subsequent mineralization (303). The mature osteoblasts are ultimately developed into either osteocytes, another type of bone cells that become embedded in the mineralized matrix, or bone lining cells covering the bone surface (301).

Osteoclasts are bone-resorbing cells that are derived from hematopoietic cells of monocyte/macrophage lineage (307). Osteoclasts are characterized by distinctive morphology of multinuclearity and high expression of tartrate-resistant acid phosphatase (TRAP) and Cathepsin K, two enzymes important for osteoclast resorption activity (308). In order for bone resorption to occur, osteoclasts must tightly adhere to the mineralized bone surface to form an isolated compartment

named the sealing zone, which encloses the resorption site, surrounded by convoluted membrane area called the ruffled border. The sealing zone contains protons and proteins that can acidify the compartment, then dissolve the mineral and eventually digest the organic compounds of bone (301, 308).

Osteoclast differentiation is a critical biological process that determines the level of bone resorption, and many cytokines and growth factors are involved in the regulation of this process (308). M-CSF (macrophage colony stimulating factor) is an essential cytokine required for generation of functional osteoclasts. Mice carrying the inactivating mutation in *Csf1* gene coding for M-CSF have osteoclast-deficient osteopetrosis (dense bones) (309). RANKL (receptor activator of nuclear factor kappa-B ligand) is another key cytokine required for osteoclast survival and differentiation. RANKL, encoded by the gene, *TNFSF11*, is a type II membrane protein of the TNF superfamily (308). Mutation of the human *TNFSF11* gene causes osteoclast-poor osteopetrosis (310). In addition, administration of RANKL in mice results in increased bone resorption (311). Furthermore, genetic ablation of *tnfsf11* gene in mice also causes osteopetrosis due to complete lack of osteoclasts (312). The binding of RANKL to its receptor RANK is negatively regulated by the soluble decoy receptor osteoprotegerin (OPG) that is secreted by osteoblasts. Loss of functional OPG in mice leads to osteoporosis (brittle bones) caused by excessive bone resorption due to up-regulated osteoclast activity (313, 314). The ratio of OPG: RANKL produced by osteoclasts may determine the degree of osteoclast differentiation and function (315).

RANKL and RANK are expressed on the cell surface of osteoblasts and osteoclast precursors, respectively. Thus, the binding of RANKL to RANK requires the direct contact between osteoblasts and osteoclast precursors (Figure 1.6). The binding activates the signalling pathway that promotes the differentiation of osteoclast precursors into the large and multinucleated osteoclast cells (308). Therefore, although bone formation and bone resorption occur independently at distinct locations, molecular communication exists between

osteoblasts and osteoclasts. Bone formation and bone resorption must be carefully balanced and coordinated for proper skeletal development and maintenance.

1.9 Thesis Objectives

Choline kinase (CK) catalyzes the first reaction of the CDP-choline pathway, also known as the Kennedy pathway, to produce phosphatidylcholine (PC) (17), the most abundant phospholipid in mammalian cellular membranes (2). CK is a cytosolic enzyme that phosphorylates choline to phosphocholine (PCho) (37). CK exists in three isoforms in mammalian cells: CKα1, CKα2 and CKβ, which are encoded by the *Chka* and *Chkb* genes, respectively (43, 44). Although there was no previous study demonstrating the direct connection between CK and bone biology, its substrate choline (112-114), reaction product PCho (120, 121) and pathway product PC (130, 131), have all been implicated in skeletal development.

Thesis objective 1:

In 2006, mice with a spontaneous genomic deletion within the *Chkb* gene were identified. The *Chkb*$^{-/-}$ mice develop hindlimb muscular dystrophy and have neonatal forelimb bone deformation. Previous work in our lab has demonstrated that impaired PC biosynthesis and mitochondrial dysfunction contribute to the muscular dystrophy phenotype in *Chkb*$^{-/-}$ mice (106, 108). However, the reason for the forelimb bone deformity was still unknown. **Our first objective was to investigate the mechanisms involved in the neonatal forelimb bone deformation in *Chkb*$^{-/-}$ mice**. In Chapter 2, we first characterized the forelimb bone deformation phenotype in *Chkb*$^{-/-}$ mice by skeletal staining and Micro-CT imaging. We also stained the embryonic forelimbs to observe if the deformity occurs before birth. Next, to determine whether or not the deformed radius and ulna of *Chkb*$^{-/-}$ mice have endochondral bone formation defects, we evaluated the growth plate structure, examined chondrocyte proliferation and differentiation, investigated the cartilage matrix degradation and mineralization, as well as osteoclast recruitment to cartilage/bone interface, by various techniques including

histology, immunohistochemistry and quantitative real-time PCR. Lastly, to see if the absence of *Chkb* could affect the CDP-choline pathway in cartilage, we measured the mass of various choline metabolites in the pathway including choline, PCho and PC by liquid chromatography-mass spectrometry.

Thesis objective 2:

Osteoblasts are cells that are responsible for the synthesis and mineralization of bone during bone formation and later bone remodeling (203-205). There is limited knowledge about the role of CK and PC biosynthesis in human osteoblasts. **Our second objective was to characterize PC metabolism in human osteoblasts and explore the potential relationship between CK and the function of human osteoblasts.** In Chapter 3, we utilized two commonly used human osteoblasts models, primary human osteoblasts (HOB) and human osteosarcoma MG-63 cells. By metabolic radiolabeling, we determined the PC sources, evaluated if CDP-choline pathway is the only functional route for PC biosynthesis, and assessed if CK catalyzes the rate-limiting step for PC biosynthesis in human osteoblasts. We also characterized and compared CK expression and activity in HOB cells and MG-63 cells by immunoblotting and enzymatic activity. CKα has been shown to play a key role in cancer cell proliferation and tumor development (98, 135-137). Thus, we silenced *Chkα* by siRNA (small interfering RNA) in MG-63 cells to investigate if this could affect the human osteosarcoma cell function. By liquid chromatography-mass spectrometry, we first quantified the pool sizes of various choline metabolites in CDP-choline pathway after siRNA treatment. We then examined if knockdown of CKα could affect cell growth and proliferation in MG-63 cells. Lastly, we inhibited CK activity using small-molecule compounds to investigate the effect on the mineralization capacity of MG-63 cells.

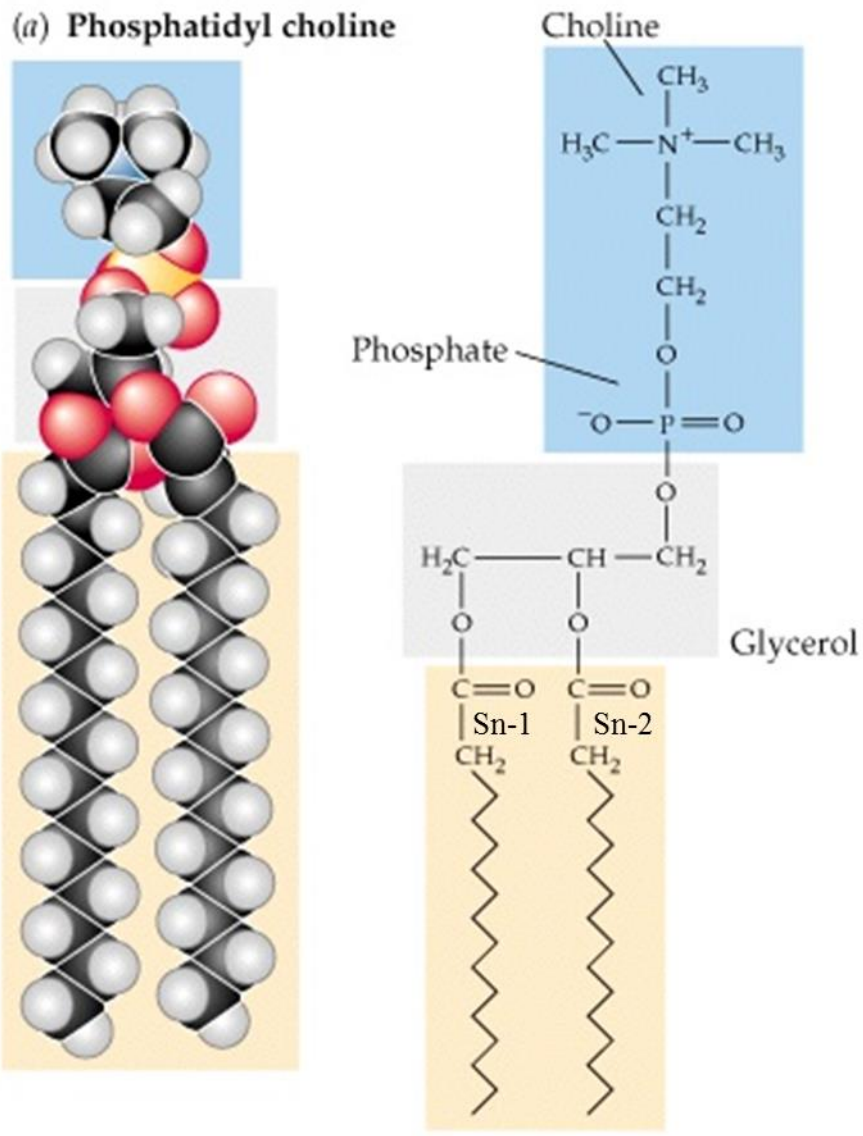

Figure 1.1 Molecular drawing of a typical phosphatidylcholine structure. Choline head group, glycerol backbone, phosphodiester linkage and fatty acyl-chains at the sn-1 and sn-2 position are indicated in the phosphatidylcholine structure. Adapted from Purves *et al*., Life: The Science of Biology, 6th Edition, by Sinauer Associates, Inc.

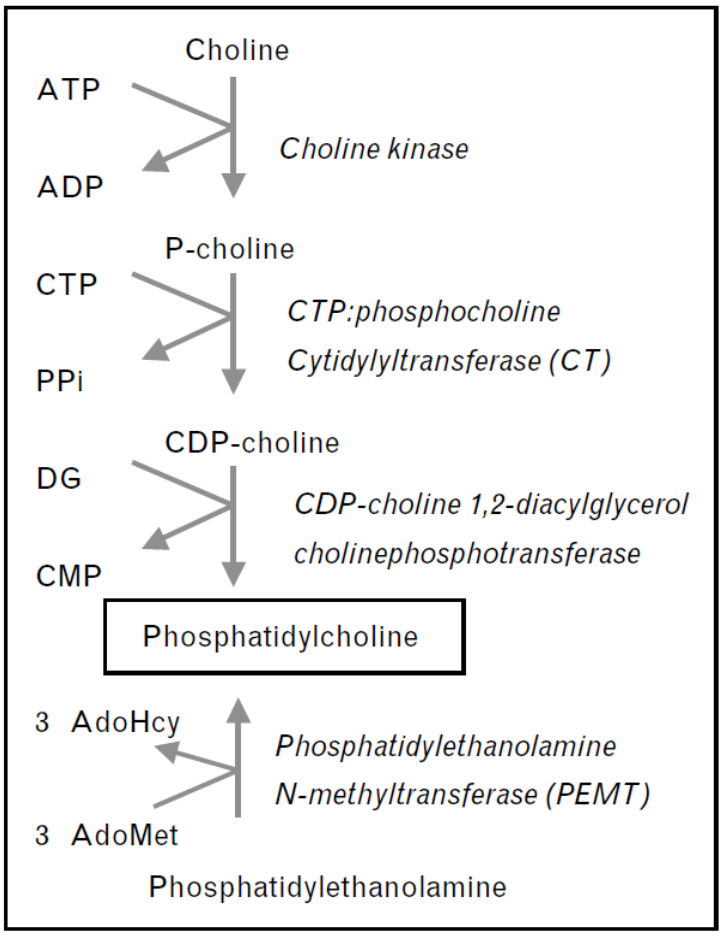

Figure 1.2 Two pathways responsible for phosphatidylcholine biosynthesis. Phosphatidylcholine (PC) are synthesized through two distinctive pathways. The cytidine diphosphate (CDP)-choline pathway (indicated on the top of the figure), requires choline as the initial substrate to produce PC. The phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway (indicated on the bottom of the figure), generates PC via three sequential methylations of phosphatidylethanolamine (PE), with *S*-adenosylmethionine (AdoMet) as the methyl donor. Other definitions include: ATP, adenosine triphosphate; ADP, adenosine diphosphate; CTP, cytidine triphosphate; PPi, pyrophosphate; DG, diacylglycerol; CMP, cytidine monophosphate; AdoHcy, *S*-adenosylhomocysteine. Adapted from Vance., Curr. Opin. Lipidol, 19 (2008) 229-234.

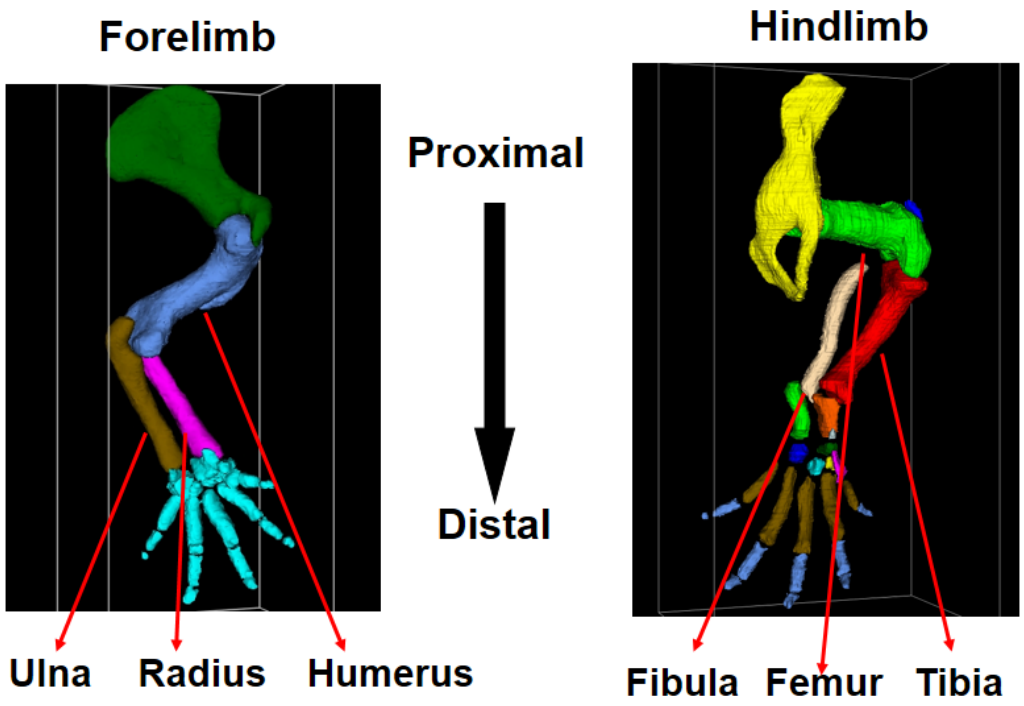

Figure 1.3 Schematic representation of various long bones in the murine forelimb and hindlimb. Humerus in forelimb and femur in hindlimb are proximal skeletal elements, whereas radius/ulna in forelimb and tibia/fibula in hindlimb are distal skeletal elements. Figure created by Interactive 3D Mouse Limb Anatomy Atlas designed by MRC National Institute for Medical Research (London, UK).

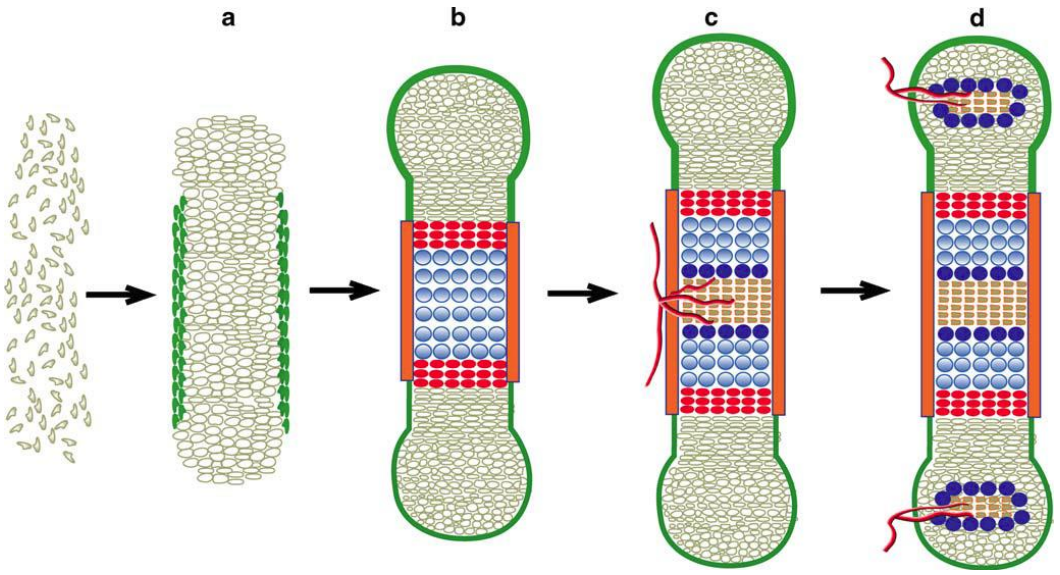

Figure 1.4 Schematic representation of endochondral bone formation. (a) Mesenchymal cells condense. (b) Chondrocytes are differentiated into distinctive subpopulations of resting/proliferating chondrocytes (grey), prehypertrophic chondrocytes (red), and hypertrophic chondrocytes (blue), which are surrounded by the perichondrium (green) or periosteum (orange). (c) Vascular invasion initiates mineralization in the center of the skeletal elements to form the primary ossification centers. (d) The secondary ossification centers are formed at the two ends of the bone, above the growth plates. Adapted from Wuelling *et al.*, Pediatr. Nephrol, 25 (2010) 625–631.

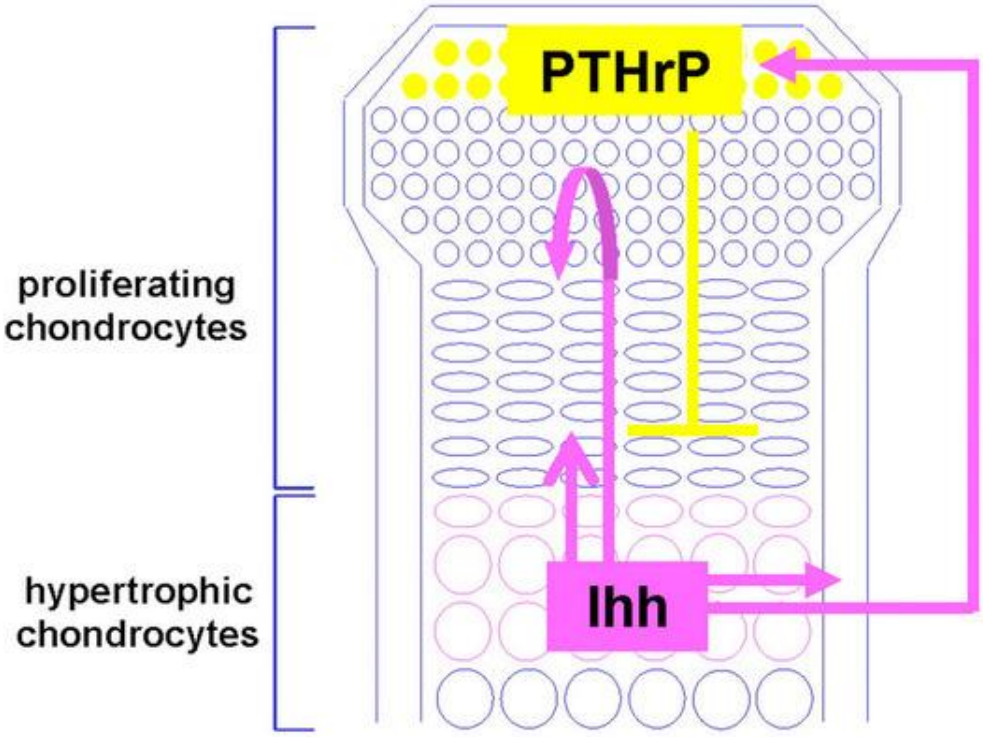

Figure 1.5 Schematic diagram of Indian hedgehog / parathyroid hormone-related protein signalling loop. Parathyroid hormone-related protein (PTHrP) is synthesized by perichondrial cells and chondrocytes located at the ends of long bones. PTHrP acts on its receptors to keep the chondrocytes proliferating and slow the differentiation of prehypertrophic and hypertrophic chondrocytes and, thereby, to delay the production of Indian hedgehog **(**Ihh). When chondrocytes eventually undergo hypertrophy, they secrete Ihh, which in turn stimulates the synthesis of PTHrP to increase the rate of chondrocyte proliferation. Adapted from Kronenberg., BoneKEy-Osteovision, 2 (2005) 7-15.

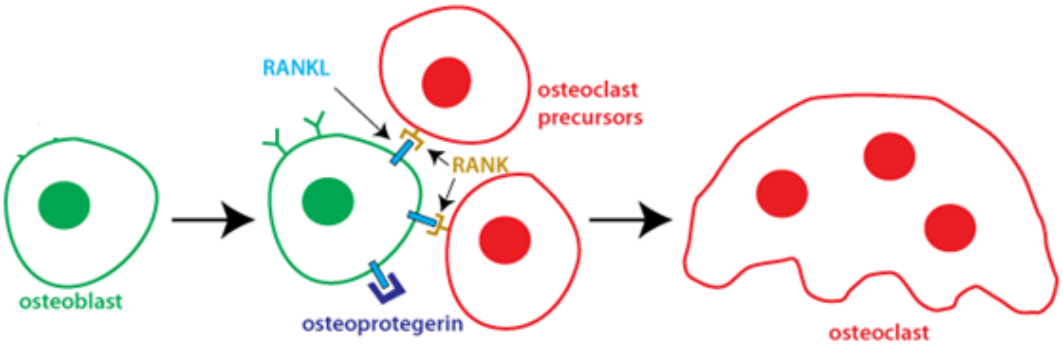

Figure 1.6 Osteoclast differentiation. Osteoclast differentiation requires direct contact between osteoblasts and osteoclast precursors. Osteoblasts express RANKL (receptor activator of nuclear factor kappa-B ligand) on the extracellular surface of their plasma membranes, and osteoclast precursors express the receptor for RANKL, RANK, on their cell surfaces. Binding of RANKL to RANK stimulates the osteoclast differentiation but the binding is inhibited by a circulating decoy receptor osteoprotegerin (OPG) secreted by osteoblasts.

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Chapter 2

Choline Kinase Beta is Required for Normal Endochondral Bone Formation

2.1 Introduction

Choline kinase (CK) catalyzes the first reaction of the CDP-choline pathway, also known as the Kennedy pathway, for synthesis of phosphatidylcholine (PC), one of the major phospholipids in mammalian cellular membranes (1, 2). CK is a cytosolic enzyme, present in all tissues and converts choline to phosphocholine (PCho) (3). CK exists in three isoforms in mice: CKα1 and CKα2 encoded by *Chkα* gene, and CKβ encoded by *Chkb* gene (4). Active CK consists of either homo- or hetero-dimeric forms; neither isoform is active in its monomeric form (4).

Previous work demonstrated that *Chkα* null mice die very early during embryonic development, indicating the importance of CK for embryonic development (5). In 2006, mice with a spontaneous 1.6 kb intragenic deletion within the *Chkb* gene were identified (6). The *Chkb*$^{-/-}$ mice have a rostral-to-caudal gradient of severity with minor forelimb but severe hindlimb muscular dystrophy. Previous work has shown that impaired PC biosynthesis and mitochondrial dysfunction contribute to the muscular dystrophy phenotype in *Chkb*$^{-/-}$ mice (7). *Chkb*$^{-/-}$ mice also display striking bone deformation in the forelimb. However, the reason for the forelimb bone deformity had not been investigated.

The limb long bones form by endochondral ossification, during which a cartilage template is first formed and is subsequently replaced by bone tissue (8-12). Endochondral bone formation begins with mesenchymal cell condensation that pre-figures the future shape of the bones. The cells in these mesenchymal condensations differentiate into chondrocytes, the major cell type in cartilage. This differentiation process is mainly regulated by the transcription factor Sox9. The chondrocytes then undergo proliferation that results in parallel columns of dividing cells. These dividing cells assume a flattened shape and form columns that resemble stacks of coins with a distinct orientation. The cells then stop dividing, exit from the cell cycle, increase in size and become mature and

hypertrophic. The hypertrophic chondrocytes then secrete a specialized extracellular matrix (ECM) which is eventually remodeled and digested by proteases. Two essential matrix metalloproteinases (MMPs), MMP9 and MMP13, are involved in the degradation of cartilage ECM. Matrix degradation can facilitate the recruitment of blood vessels, as well as osteoblast and osteoclast cells to invade the hypertrophic cartilage for replacing it with mineralized bone tissue. As bone enlarges further, a so-called secondary ossification center is established by a similar mechanism at the two ends of the bones. The cartilage structure between the two ossification centers is known as the growth plate. The growth plate consists of distinct layers including the resting zone, proliferation zone and hypertrophic zone.

In this study, we have demonstrated that *Chkb* plays an important role in endochondral bone formation and growth plate physiology. The endochondral bone formation defects and growth plate phenotypes of *Chkb*$^{-/-}$ mice significantly contribute to the forelimb bone deformity.

2.2 Experimental Procedures

2.2.1 Mouse breeding and genotyping

Chkb$^{-/-}$ mice in C57BL/6J background were originally generated at the Jackson Laboratory (Bar Harbor, Maine, USA) (6). All animal procedures were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. Wild-type littermates were used as controls. Heterozygous mice were bred with each other so that litters and embryos of three genotypes were obtained. PCR genotyping was performed using DNA from digested tail samples. Two separate genotyping programs were used to amplify both the wild-type *Chkb* allele between exons 5 and 9 and the truncated *Chkb* allele between exons 3 and 11. The mutation identified in *Chkb*$^{-/-}$ mice is a 1.6 kb genomic deletion between exon 3 and intron 9 (6). The primers used for genotyping were purchased from Integrated DNA Technologies and the sequences are included in Table 2.1.

2.2.2 Skeletal stains and Micro-CT imaging

For staining of neonatal mouse skeleton and embryonic limb bones by Alizarin Red and Alcian Blue, the tissues were fixed overnight in 95% ethanol followed by overnight fixation in acetone. The tissues were then placed in staining solution for one week (0.05% Alizarin Red, 0.015% Alcian Blue, 5% acetic acid in 70% ethanol). The tissues were then cleared in 2% KOH followed by 1% KOH until they were almost clean. Images of stained bones were obtained with an Olympus SP-57OUZ camera. The lengths of limb bones were measured in four littermate pairs using a dissecting microscope with a ruler. For Micro-CT imaging, intact mice and mouse forelimbs were scanned by Micro-CT (Skyscan 1076, Kontich, Belgium) at 9 μm resolution with 100 kV, 100 μA, and power of 10 W. Projected images of the samples were reconstructed using vendor supplied software (Nrecon 1.6.1.5, SkyScan NV, Kontich, Belgium).

2.2.3 Serum biochemistry

Serum calcium level was measured using the QuantiChrom^{TM} calcium assay kit (BioAssay Systems). Insulin-like growth factor 1 (IGF-1) concentration in serum was measured using an IGF-1 immunoassay kit (R&D Systems). Inorganic phosphate (Pi) levels in serum, cells and tissues were measured by phosphate colorimetric assay kit (Abcam).

2.2.4 Histological analysis, tartrate-resistant acid phosphatase (TRAP) staining and immunohistochemistry

The long bones from new-born (P0) mice and embryos were dissected and fixed in 4% paraformaldehyde overnight. The tissues were then embedded in paraffin and 5 μm thick sections were prepared. Sections were de-waxed in xylene followed by graded dilutions of ethanol (100% twice, 95% once and 70% once) and finally water. For Safranin-O/fast green staining, tissue sections were stained in 0.01% fast green for 30 min, dipped in 1% acetic acid solution, then stained in 0.1% Safranin-O for 5 min. For von Kossa and van Gieson staining, the sections were stained in 1% silver nitrate solution, washed in distilled water, treated with 5% sodium thiosulfate, washed in tap water and finally van Gieson stain was applied. Tissue sections were dehydrated and coverslips were mounted using a xylene-based mounting solution. Growth plate morphology was examined with a Leica DMRA2 microscope equipped with a Retiga EX camera. The length of the hypertrophic zone was measured using OpenLab 4.0.4 software (13-15).

For immunohistochemistry, sections were incubated in 3% H_2O_2 for 15 min at room temperature, followed by antigen retrieval by incubation in 0.1% Triton X-100 for 13 min, followed by blocking with 5% goat or rabbit serum in phosphate-buffered saline. Sections were incubated with primary antibodies overnight at 4 ℃ after which secondary antibodies were applied according to manufacturers' recommendations. Primary antibodies were: goat polyclonal anti-human Sox9 (AF3075, R&D Systems) and mouse monoclonal anti-mouse

proliferating cell nuclear antigen (PCNA) (2586S, Cell Signalling). Incubation with horseradish peroxidase-conjugated secondary antibody (Abcam) was followed by colorimetric detection with the substrate diaminobenzidine (DAB) (Dako Inc). Three independent stainings were performed and representative images are shown.

For TRAP staining, bone tissue sections from 7-day-old (P7) mice were stained to assess osteoclast numbers using a leukocyte acid phosphatase kit (Sigma). The staining was quantified by counting the number of positive foci along the chondro-osseous junction.

2.2.5 Primary culture of chondrocytes

Primary chondrocytes were prepared from long bones of embryonic 15.5-day-old (E15.5) mouse embryos, as previously described (16). Briefly, the long bones were dissected and incubated at 37 ℃ for 15 min in 0.25% trypsin-EDTA, followed by digestion at 37 ℃ with 3 mg/ml collagenase P for 2 h in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). The cell suspensions were filtered through a 40 mm cell strainer (Fisher Scientific), washed, counted and plated at 37 ℃ in 6-well plates with media containing 2:3 DMEM:F12, 10% FBS, 0.5 mM L-glutamine, and penicillin/streptomycin (25 units/ml).

2.2.6 RNA isolation and quantitative real-time PCR analysis

RNA was isolated directly from either E15.5 embryonic bones or primary chondrocytes. For embryonic bones, E15.5 radius and ulna were dissected free from connective tissue. The bones were then placed into RNA stabilization reagent (Qiagen) and passed through a series of syringes with 21G, 23G and 25G needles (BD Biosciences) to break the tissues. The RNA in the homogenate was purified with RNeasy Micro Kit according to the manufacturer's instructions (Qiagen). For primary chondrocytes, RNA was isolated using TRIzol reagent, then reverse-transcribed by oligo (dT) and Superscript II reverse transcriptase

(Invitrogen). Quantitative real-time PCR was performed using a Rotor-Gene 3000 instrument and data were analyzed using the Rotor-Gene 6.0.19 software (Montreal Biotech). Cyclophilin mRNA was used to normalize gene expression. The primers were purchased from Integrated DNA Technologies and the sequences are included in Table 2.1.

2.2.7 Immunoblot analyses and choline kinase assay

For immunoblotting, primary chondrocyte cells were harvested in buffer containing 10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (1:100 dilution, P8340, Sigma). The cell extracts were centrifuged at 348,000 $\times g$ for 15 min at 4 ℃. The protein content of the supernatant (cytosol) was quantified by the Bradford procedure (17). Proteins (50 μg/lane) were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS, and immunoblotted with the relevant primary antibodies. The following antibodies were used: rabbit polyclonal anti-mouse CKβ (dilution 1:1000; a gift from Dr. K. Ishidate, Teikyo Heisei University, Japan) and mouse monoclonal anti-rat Phospho1 (dilution 1:500, ab90581, Abcam). Antibodies raised against either α-tubulin or β-actin (Abcam) were used for loading controls.

Total CK activity was determined in the cytosolic fraction as described (18), with minor modifications. Briefly, the supernatants were incubated in a final volume of 100 μl buffer containing 0.1 M Tris-HCl, pH 8.75, 10 mM ATP, 15 mM $MgCl_2$, and 0.25 mM [^{3}H] choline chloride (10.5 μCi/ml) at 37 ℃ for 30 min. The product PCho was separated using an AG1-X8 (200-400 mesh, OH- form) column (Bio-Rad).

2.2.8 Gelatin zymography

To determine MMP9 activity, primary chondrocyte cells were harvested in MMP lysis buffer (120 mM Tris, 0.1% Triton X-100, 0.01% NaN_3, 5% glycerol at pH 8.7). Proteins (10 μg/lane) were electrophoresed on SDS-polyacrylamide gels

co-polymerized with gelatin (2 mg/ml). Distilled water and 20 µg proteins from the chondrogenic cell line ATDC5 (Sigma) were loaded onto the gel as negative and positive controls, respectively. Following electrophoresis, the gel was washed 4 times with 2.5% Triton X-100 for 15 min. MMP9 activity was developed by incubating the gel overnight at 37 ℃ in incubation buffer (50 mM Tris, 5 mM $CaCl_2$, 0.15 M NaCl, 0.5 mM NaN_3, pH 7.6) after which the gel was stained with Coomassie blue.

2.2.9 Mass-spectrometric analysis of lipids and water-soluble choline metabolites

Lipids were extracted from primary chondrocytes (100 µg protein) according to the method described in (19). Phospholipid internal standards were used for quantification of the lipid species and were added at the stage of lipid extraction. The lipids were quantified using liquid chromatography-mass spectrometry (LC-MS) as described (20). The acyl residues of the lipid species were quantified using fragmentation analysis (21). The water-soluble choline metabolites were analyzed by LC-MS/MS as described previously (22).

2.2.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism software 6.0. Data are mean values ± standard error of the mean (SEM). Statistical significance of differences ($P < 0.05$) was determined by the student's *t* test.

2.3 Results

2.3.1 Deformation of the radius and ulna in *Chkb*$^{-/-}$ mice

Figure 2.1A shows the striking outward forelimb bone deformation of 7-day-old (P7) *Chkb*$^{-/-}$ mice. The forelimb bone deformation phenotype was also observed in newborn mice, and an initial characterization of this phenotype has been described (6). To further characterize the forelimb bone deformity, the whole body and forelimbs of newborn (P0) *Chkb*$^{-/-}$ mice and wild type littermates were stained with Alizarin Red & Alcian Blue and viewed by Micro-CT imaging. No gross skeletal abnormalities were observed, except for the obvious deformations of the radius and ulna of the forelimb (Figures 2.1B, C, D and E). No deformity of other limb long bones, including the humerus of the forelimb, was observed (Figure 2.1C and Figure 2.2C). To determine if the neonatal bone deformity persisted throughout the postnatal stage, the radius and ulna of 30-day-old (P30) mice were scanned by Micro-CT. The reconstructed image shows that the bone deformations in *Chkb*$^{-/-}$ mice become more severe over time and that the radius and ulna are curved almost to 90 degrees (Figure 2.1F). Together, our observations revealed that the forelimb bone deformations in *Chkb*$^{-/-}$ mice are restricted to the radius and ulna and the deformities become more severe at later stages of development.

2.3.2 The radius and ulna in *Chkb*$^{-/-}$ mice are shorter than in *Chkb*$^{+/+}$ mice and *Chkb*$^{-/-}$ mice have growth retardation

To further characterize the limb long bones, the radius, ulna and humerus from the forelimb, and the tibia and femur from the hindlimb, of newborn mice were stained with Alizarin Red and Alcian Blue. In addition to the deformities, the lengths of the radius and ulna in neonatal *Chkb*$^{-/-}$ mice were significantly shorter than in *Chkb*$^{+/+}$ mice, whereas the lengths of the humerus, tibia and femur were not different (Figures 2.2A, C). However, in 21-day-old (P21) *Chkb*$^{-/-}$ mice, the humerus, tibia and femur were slightly shorter than in *Chkb*$^{+/+}$ mice while the

lengths of the radius and ulna were dramatically reduced (Figure 2.2B). Shorter limb long bones in postnatal *Chkb*$^{-/-}$ mice implicates retarded growth of these mice. Indeed, *Chkb*$^{-/-}$ mice are smaller (Figure 2.1A), and they gained significantly less body weight before weaning (Figure 2.3A). The circulating level of IGF-1, an important regulator of postnatal skeletal development (23, 24), was approximately 50% lower in *Chkb*$^{-/-}$ mice than in *Chkb*$^{+/+}$ mice (Figure 2.3B), which might explain the smaller skeleton and growth retardation in *Chkb*$^{-/-}$ mice.

2.3.3 The radius and ulna in *Chkb*$^{-/-}$ mice are deformed during embryonic development

The deformities of the radius and ulna in *Chkb*$^{-/-}$ mice are evident at birth, raising the possibility that their deformations occur during embryonic development. We, therefore, isolated and stained the forelimbs from E13.5 to E17.5 mice. We observed deformations of the radius and ulna of *Chkb*$^{-/-}$ mice during late embryonic development (by E17.5) (Figure 2.4). Furthermore, the radius and ulna in *Chkb*$^{-/-}$ mice were shorter than in wild-type littermates as early as E14.5 stage, suggesting that *Chkb*$^{-/-}$ mice have defects in embryonic cartilage development and that the bone shortening precedes the deformity.

2.3.4 Unaltered *Hoxa11* and *Hoxd11* gene expression in the radius and ulna of *Chkb*$^{-/-}$ mice

We observed that bone deformities are only restricted in the radius and ulna and they occur prior to birth (Figure 2.1 and Figure 2.4). These observations suggest that the bone deformations may be caused by abnormal limb patterning, which takes place primarily during early embryonic development and regulates the formation of specific skeletal elements of the limb (8). The formation and pattering of limbs are guided by *Hox* genes. Specific combinations of mutations in *Hox* genes result in severely deformed skeletal elements in the limb (25). The formation and patterning of the radius and ulna are tightly regulated by Hoxa11 and Hoxd11. Inactivation of murine *Hoxa11* and *Hoxd11* completely disrupts

formation of radius and ulna of the forelimb (26, 27). To determine if the deformations of the radius and ulna in *Chkb*$^{-/-}$ mice were due to altered *Hoxa11* and *Hoxd11* gene expression, we performed quantitative real-time PCR analysis of E15.5 embryonic bones and primary chondrocytes from *Chkb*$^{-/-}$ and *Chkb*$^{+/+}$ mice but did not detect any differences in expression of these two genes (Figure 2.5). This observation suggests that the deformities of the radius and ulna are probably not caused by abnormal limb patterning. This conclusion is also supported by the appearance of the deformities during late embryonic development (Figure 2.4), long after patterning has occurred.

2.3.5 The radius and ulna in *Chkb*$^{-/-}$ mice have growth plate defects

The presence of shorter embryonic and neonatal radius and ulna in the *Chkb*$^{-/-}$ mice suggests that the biology of their growth plates might be abnormal. Therefore, we studied the growth plate organization of the radius and ulna in P0 and E16.5 mice by Safranin O/fast green staining. Figure 2.6 shows representative images and quantification of the staining. The staining clearly shows that both the radius and ulna of *Chkb*$^{-/-}$ mice contain expanded hypertrophic zones (Figures 2.6A, B). The ratio of hypertrophic zone length / total growth plate length also indicates that hypertrophic zones of the *Chkb*$^{-/-}$ mice are longer and enlarged compared to those in *Chkb*$^{+/+}$ mice (~1.5 fold higher for the radius and ~2-fold higher for the ulna) (Figures 2.6C, D). Moreover, the radius and ulna of E16.5 *Chkb*$^{-/-}$ mice also contained disorganized proliferation zones with loss of columnar organization and irregular chondrocyte morphology (Figures 2.6E, F). Together, these observations clearly show that the radius and ulna of *Chkb*$^{-/-}$ mice have defects in growth plate development.

2.3.6 Impaired chondrocyte differentiation and decreased chondrocyte proliferation in the radius and ulna from *Chkb*$^{-/-}$ mice

The growth plate phenotypes observed in the radius and ulna of *Chkb*$^{-/-}$ mice suggest that endochondral bone formation is defective. We, therefore,

examined chondrocyte differentiation and proliferation by immunohistochemistry of Sox9 and proliferating cell nuclear antigen (PCNA) expression in paraffin sections of E15.5 bones. Sox9 is an early chondrogenic marker that regulates chondrocyte differentiation (28). The growth plates of both the radius and ulna of *Chkb*$^{-/-}$ mice exhibited significantly less Sox9 and PCNA expression than did of *Chkb*$^{+/+}$ mice (Figures 2.7A, B, D, and E). Quantification of the percentage of Sox9- and PCNA-expressing cells is shown in Figure 2.7G and Figure 2.7H, respectively. These data demonstrate that chondrocyte differentiation and proliferation of the radius and ulna are decreased in *Chkb*$^{-/-}$ mice. However, chondrocyte differentiation and proliferation of the humerus appear to be normal since Sox9 and PCNA expression is comparable to that in wild-type littermates (Figures 2.7C, F, G and H).

2.3.7 *Mmp9* and *Mmp13* expression is attenuated in the radius and ulna of *Chkb*$^{-/-}$ mice

Several MMPs are essential for the cleavage of cartilage ECM and replacement of cartilage by bone tissue. Loss of specific MMPs, such as MMP9 and MMP13, impairs matrix degradation and expands the hypertrophic zone in the growth plate (29-31). Since the hypertrophic zones in the radius and ulna of *Chkb*$^{-/-}$ mice were enlarged, we studied MMP9 and MMP13. We first performed quantitative real-time PCR gene expression analysis of the mRNAs encoded by *Mmp9* and *Mmp13* and observed significantly lower levels of *Mmp9* and *Mmp13* mRNAs in both embryonic bones and primary chondrocytes in the radius and ulna of *Chkb*$^{-/-}$ mice compared to wild-type mice (Figures 2.8A, B). We also performed gelatin zymography assays for MMP9 activity, using primary chondrocytes isolated from the radius and ulna of E15.5 mice. MMP9 digests gelatin and exists in two forms: an uncleaved pro-form and a cleaved mature form (32). Thus, MMP9 activity can be visualized as two bands after staining and destaining the gel. This assay showed that chondrocytes from the radius and ulna of *Chkb*$^{-/-}$ mice contained markedly less MMP9 activity than did those from *Chkb*$^{+/+}$ mice (Figure 2.8C). These data suggest that the radius and ulna from *Chkb*$^{-/-}$ mice have

impaired MMPs activity that leads to decreased cartilage ECM degradation.

2.3.8 Recruitment of osteoclasts to the chondro-osseous junction in the radius and ulna

Osteoclasts play an important role in endochondral ossification by cartilage resorption and the conversion of cartilage matrix to bone matrix (12, 33). Mice that are deficient in osteoclast production have enlarged hypertrophic zones in their growth plates (34). Consequently, we determined if the growth plate phenotypes observed in *Chkb*$^{-/-}$ mice were associated with impaired cartilage resorption by staining P7 radius and ulna bone sections for tartrate-resistant acid phosphatase (TRAP), a known marker of osteoclasts (33). TRAP staining of the radius and ulna of *Chkb*$^{-/-}$ mice along the chondro-osseous junction was significantly less than that of the *Chkb*$^{+/+}$ mice (Figures 2.9A, B, C and D). The number of TRAP-positive foci in the radius and ulna was approximately 50% less in the *Chkb*$^{-/-}$ mice than in the *Chkb*$^{+/+}$ mice (Figures 2.9E, F). These data suggest that the recruitment of osteoclasts to the cartilage/bone junction is impaired in the radius and ulna of *Chkb*$^{-/-}$ mice.

2.3.9 Primary ossification of the radius and ulna

The aforementioned endochondral bone formation defects in the radius and ulna of *Chkb*$^{-/-}$ mice, including impaired ECM degradation and cartilage resorption, likely lead to delayed chondrocyte mineralization. We, therefore, determined if cartilage mineralization was altered by performing von Kossa staining of E15.5 *Chkb*$^{-/-}$ and wild-type mice bone sections from the radius and ulna to examine the formation of primary ossification centers. Figure 2.10 shows that the mineralized area in the bone center of both the radius and ulna of *Chkb*$^{-/-}$ mice was smaller than in wild-type mice and that the zone of unmineralized cartilage was larger. These data indicate that formation of the primary ossification center of the radius and ulna of *Chkb*$^{-/-}$ mice is delayed. The enlargement of the hypertrophic zone and delayed cartilage mineralization are reminiscent of

osteomalacia, a disease characterized by impaired bone mineralization, secondary to reduced plasma calcium levels (35). However, serum calcium levels were not lower in *Chkb*$^{-/-}$ mice compared to *Chkb*$^{+/+}$ mice (Figure 2.12B).

2.3.10 Biochemical analyses in primary chondrocytes

To confirm that CKβ had been eliminated in the *Chkb*$^{-/-}$ mice, limb long bones from E15.5 mice were dissected free of soft tissue, and primary chondrocytes were isolated. Immunoblotting confirmed the absence of CKβ in *Chkb*$^{-/-}$ mice (Figure 2.11A). Moreover, total CK activity was approximately 80% lower in chondrocytes from all major long bones of *Chkb*$^{-/-}$ mice compared to wild-type mice (Figure 2.11B). Because CK catalyzes the first reaction of the CDP-choline pathway for the synthesis of PC, we quantified the levels of choline metabolites and the major phospholipids in *Chkb*$^{-/-}$ and *Chkb*$^{+/+}$ chondrocytes. Primary chondrocytes isolated from the combined radius and ulna were used for quantification of amounts of these metabolites by mass spectroscopy. Chondrocytes from the radius and ulna of *Chkb*$^{-/-}$ mice contained approximately 75% less PCho than those from *Chkb*$^{+/+}$ mice whereas the amount of choline was not different (Figure 2.11C). The amount of the phospholipid product, PC, was ~10% lower in chondrocytes from *Chkb*$^{-/-}$ mice compared to *Chkb*$^{+/+}$ mice; amounts of other phospholipids were unchanged by CKβ deficiency (Figure 2.11D). Thus, the absence of CKβ in *Chkb*$^{-/-}$ mice only slightly reduces the amount of PC but significantly decreases PCho production in cartilage.

2.4 Discussion

These data show that the forelimb bone deformations in *Chkb*$^{-/-}$ mice are limited to the radius and ulna, and that the deformities occur before birth. Moreover, the radius and ulna of *Chkb*$^{-/-}$ mice have abnormal growth plate physiology and endochondral bone formation, including expanded hypertrophic zones, impaired chondrocyte differentiation and proliferation, decreased cartilage ECM digestion and osteoclast recruitment, and delayed formation of the primary ossification center.

2.4.1 Deformations of the radius and ulna of *Chkb*$^{-/-}$ mice are probably not caused by abnormal limb patterning

We observed that forelimb bone deformity of *Chkb*$^{-/-}$ mice is restricted to the radius and ulna (Figure 2.1), which is consistent with the initial characterization of *Chkb*$^{-/-}$ mice (6). Furthermore, we found that radius and ulna of *Chkb*$^{-/-}$ mice are deformed during embryonic stage (Figure 2.4), implicating possible abnormal limb patterning. The patterning of radius and ulna during embryonic development is tightly controlled by *Hoxa11* and *Hoxd11* genes, and depletion of both *Hoxa11* and *Hoxd11* genes in mice completely prevents the formation of the radius and ulna (26, 27). However, we did not observe gene expression alteration of these two genes (Figure 2.5), suggesting that they are not involved in the radius and ulna deformation phenotype of *Chkb*$^{-/-}$ mice.

The initial phenotype characterized in *Chkb*$^{-/-}$ mice was muscular dystrophy; *Chkb* mutations have been identified in humans with muscular dystrophy (36). However, no *Chkb* mutations in humans have been associated with bone disease. The radius and ulna deformations (Figure 2.1), and the growth retardation phenotype (Figures 2.2 and 2.3) in *Chkb*$^{-/-}$ mice are reminiscent of Turner syndrome, Leri-Weill dyschondrosteosis and Langer syndrome in humans, which are associated with mutations in the short stature homeobox (Shox) gene. These individuals are characterized by dwarfism, limb shortening and bowed

radius/ulna and tibia/fibula (37-39). However, mice do not express the *Shox* gene (40) but do have the *Shox2* paralog, which is also expressed in humans (41). *Shox2* deficiency in mice severely affects proximal limb development including that of the humerus and femur; in contrast, development of the radius and ulna, that are distal skeletal elements, is normal (42-44). In *Chkb*$^{-/-}$ mice, the most prominent defects are in the radius and ulna, suggesting that the bone deformity phenotype is not linked to *Shox2* gene.

2.4.2 Radius and ulna of *Chkb*$^{-/-}$ mice have impaired replacement of cartilage by bone

The most dramatic phenotypes observed in the growth plates of the radius and ulna from *Chkb*$^{-/-}$ mice are expanded hypertrophic zones (Figure 2.6) and delayed formation of the primary ossification center (Figure 2.10). These two growth plate defects suggest that CKβ deficiency impairs the conversion of cartilage to bone tissue. Cartilage matrix degradation plays an essential role in the replacement of cartilage by bone tissue. Invasion of the ossification front, including the formation of blood vessels, as well as osteoblast and osteoclast cells, requires extensive degradation of cartilage ECM surrounding the late hypertrophic cells (12). Two matrix proteinases, MMP9 and MMP13, are mainly responsible for the degradation of cartilage ECM. Mice deficient in either *Mmp9* or *Mmp13* have expanded hypertrophic zones and delayed primary endochondral ossification due to impeded digestion of ECM (29-31). Interestingly, in *Chkb*$^{-/-}$ mice, *Mmp9* and *Mmp13* gene expression was significantly reduced in both embryonic radius and ulna, as well as in primary chondrocytes (Figures 2.8A, B). The chondrocytes isolated from the radius and ulna of *Chkb*$^{-/-}$ mice also exhibited diminished MMP9 activity *in vitro* (Figure 2.8C). These data suggest that cartilage ECM degradation is impaired in the radius and ulna of *Chkb*$^{-/-}$ mice, leading to slower conversion of cartilage into bone.

Osteoclasts are also important for establishment of primary ossification. In the absence of osteoclasts, cartilage ECM cannot be removed and there is no

invasion by the ossification front or bone deposition on cartilage remnants (12). TRAP staining revealed that the radius and ulna of *Chkb*$^{-/-}$ mice recruit fewer osteoclasts to the cartilage/bone interface (Figure 2.9), indicating decreased cartilage resorption by osteoclasts. These findings further support the conclusion that replacement of cartilage by bone in the radius and ulna is impaired in *Chkb*$^{-/-}$ mice. Previous studies showed that MMPs, particularly MMP9, are necessary for the migration of osteoclasts into hypertrophic cartilage during primary endochondral ossification (45, 46). However, we have not directly proven that reduced osteoclast recruitment in our *Chkb*$^{-/-}$ mice is due to decreased MMP9 expression and activity.

2.4.3 Radius and ulna of *Chkb*$^{-/-}$ mice have impaired chondrocyte differentiation and decreased chondrocyte proliferation

In addition to the defects in endochondral bone formation discussed above, we demonstrated that the radius and ulna of *Chkb*$^{-/-}$ mice have impaired chondrocyte differentiation and decreased proliferation since the expression of Sox9 and PCNA was reduced in the growth plates (Figures 2.7A, B, D, and E). Sox9 is an essential transcription factor that regulates the differentiation of chondrocytes from mesenchymal cells during early chondrogenesis (25). Haplo-insufficiency of *Sox9* in mice causes abnormalities in primordial cartilage including bending of the radius and ulna (47). The decreased expression of Sox9 in the radius and ulna of embryonic *Chkb*$^{-/-}$ mice might be an important factor that contributes to the bending of these bones. Moreover, a reduction in chondrocyte proliferation is consistent with the finding that bone lengths of the radius and ulna are reduced in *Chkb*$^{-/-}$ mice (Figure 2.2). Both CK and its product PCho have been implicated in cell proliferation and transformation (48-51). Production of PCho is an important event in growth factor-induced mitogenesis in fibroblasts (52-54). Moreover, increased CK activity has been detected in various cancer cells, and an elevated PCho level has been used as a biomarker for tumor diagnosis (55-58). The amount of PCho in chondrocytes from the radius and ulna of *Chkb*$^{-/-}$ mice was significantly lower than in *Chkb*$^{+/+}$ mice (Figure 2.11C). Hence, the decrease

in PCho might contribute to the growth defects in radius and ulna of *Chkb*$^{-/-}$ mice.

2.4.4 *Chkb* is the dominant isoform in chondrocytes of all the limb long bones

The reason why the bone deformities were restricted to the radius and ulna in *Chkb*$^{-/-}$ mice, but did not occur in other limb bones, is unclear. Our previous work on the muscular dystrophy phenotype in *Chkb*$^{-/-}$ mice demonstrated that CKβ is the major CK isoform in the muscle of hindlimb but not forelimb [59]. This finding provides an explanation for why the muscle damage of *Chkb*$^{-/-}$ mice is more severe in the hindlimb than in the forelimb. Total CK activity in chondrocytes isolated from all the limb long bones of *Chkb*$^{-/-}$ mice was ~80% lower than in *Chkb*$^{+/+}$ mice (Figure 2.11B), suggesting that CKβ is the dominant isoform in chondrocytes from all the limb long bones. This observation appears to eliminate the possibility that the bone defects specifically in the radius and ulna are due to different expression levels of the CK isoforms in the radius/ulna versus other limb long bones.

2.4.5 Absence of CKβ in *Chkb*$^{-/-}$ mice only slightly decreases PC but significantly reduces PCho in chondrocytes

In order to determine whether or not *Chkb* deficiency affected the amounts of choline metabolites and phospholipids in the cartilage of *Chkb*$^{-/-}$ mice, we isolated primary chondrocytes and quantified these metabolites by mass spectrometry. The PC content of chondrocytes from *Chkb*$^{-/-}$ mice was slightly lower than that of wild-type chondrocytes (Figure 2.11D). This observation was not surprising since our previous studies have shown that the PC content of most tissues of *Chkb*$^{-/-}$ mice was not altered (6). Moreover, the rate-limiting enzyme in the CDP-choline pathway for PC synthesis is CTP: phosphocholine cytidylyltransferase (CT), rather than CK (60, 61). Interestingly, two very recent human genetic studies showed that homozygous mutations in the gene coding for CT are identified in patients with spondylometaphyseal dysplasias (62, 63), a rare autosomal-recessive disorder characterized by short stature, progressive lower-

limb bowing, flattened vertebrae, metaphyseal abnormalities and visual impairment (64-66). Since CT is the regulatory enzyme in the CDP-choline pathway to synthesize PC, the authors proposed that the skeletal pathological phenotypes in those patients may be caused by attenuated PC biosynthesis due to loss-of-function mutations of CT. However, in our study, we only observed a very minor decrease of PC in chondrocytes from *Chkb*$^{-/-}$ mice. Thus, the bone defects in *Chkb*$^{-/-}$ mice are unlikely to be caused by a reduced content of PC. Nevertheless, the PCho content of chondrocytes from *Chkb*$^{-/-}$ mice was dramatically reduced, suggesting that lower PCho in cartilage is more likely to be the cause of the bone defects (Figure 2.11C). This reduction of PCho was expected since most intracellular PCho is produced from CK (2, 61).

2.4.6 Other possible roles of decreased PCho in the phenotypes of *Chkb*$^{-/-}$ mice

In addition to a role for PCho in cell proliferation, PCho has also been implicated in regulating the function of Phospho1, a recently identified phosphatase that is expressed in the mineralization surface of both bone and cartilage (67). Phospho1 plays an important role in skeletal calcification (68, 69), and *Phospho1* knockout mice have decreased bone mineral density and deformed limb bones (70). Phospho1 is a soluble cytosolic enzyme that has high phosphohydrolase activity towards PCho and phosphoethanolamine (PEA) (71, 72). PCho and PEA are the two most abundant phospho-monoesters in cartilage (73). CK also phosphorylates ethanolamine to PEA (18, 74, 75). Thus, possibly *Chkb* deficiency reduces the availability of substrates (PCho and PEA) for Phospho1 and thereby limits the ability of Phospho1 to produce sufficient inorganic phosphate (Pi) for cartilage mineralization. We observed that the amount of Phospho1 protein was higher in chondrocytes from *Chkb*$^{-/-}$ mice than in chondrocytes from *Chkb*$^{+/+}$ mice (Figure 2.12A), suggesting that a compensatory mechanism is activated in response to lower substrate levels. Although the serum level of Pi is lower in *Chkb*$^{-/-}$ mice than in wild-type mice, the Pi content of both embryonic bone tissues and primary chondrocytes was not altered by CKβ

deficiency (Figures 2.12C,D, E), suggesting that chondrocytes from *Chkb*$^{-/-}$ mice have sufficient Pi for normal mineralization. Indeed, the contribution of PCho and PEA to the total Pi pool is still unknown (68). Furthermore, other phosphorus-containing molecules such as adenosine triphosphate (ATP), adenosine diphosphate (ADP) and pyrophosphate (PPi) are also important sources of Pi, which are not hydrolyzed by Phospho1 (71). Instead, these molecules are hydrolyzed by the major phosphatase in skeletal tissue, alkaline phosphatase (ALP) (76-78). In addition, the staining and Micro-CT images of skeletons did not reveal any obvious calcification defects in *Chkb*$^{-/-}$ mice (Figure 2.1). Therefore, we propose that the delay in cartilage mineralization in the radius and ulna of *Chkb*$^{-/-}$ mice is primarily due to slower replacement of cartilage by bone rather than defects in mineralization capability.

Although the majority of PCho is produced by the CK reaction, some PCho (along with ceramide) is generated from sphingomyelin by the neutral sphingomyelinase 2 (nSMase2), which is encoded by the *Smpd3* gene. *Smpd3*-deficient mice display dwarfism, chondrodysplasia, limb bone deformation and abnormal embryonic growth plate phenotypes (79-82). These features are similar to those exhibited by *Chkb*$^{-/-}$ mice. The role that PCho and/or ceramide play in the bone phenotypes of *Smpd3*-deficient mice is still unknown.

We, therefore, propose that since PCho plays key roles in cell proliferation and skeletal mineralization (discussed above), the reduced level of PCho in chondrocytes is still the most likely underlying cause for the developmental defects in the radius and ulna of *Chkb*$^{-/-}$ mice. Future studies on tissue-specific inactivation of *Chkb,* or even *Chkα,* in cartilage, osteoblasts or osteoclasts are likely to provide additional novel insights into the role of CK in normal endochondral bone formation.

2.5 Conclusion

In conclusion, our data show that *Chkb*$^{-/-}$ mice have bone deformations specifically in the radius and ulna and that these defects occur during late embryonic development. *Chkb* deficiency in chondrocytes leads to a dramatic reduction in the amount of PCho but only a slight decrease of PC. The radius and ulna of *Chkb*$^{-/-}$ mice have several defects in endochondral bone formation including abnormal growth plate organization, decreased chondrocyte differentiation and proliferation, impaired cartilage ECM degradation, reduced osteoclast recruitment and delayed cartilage mineralization. These combined defects in cartilage development significantly contribute to the deformities of the radius and ulna. To our knowledge this is the first study to demonstrate that CKβ plays an essential role in endochondral bone formation, at least in the radius and ulna.

Primer	Forward 5'-3'	Reverse 5'-3'
Chkb exon 5-9	GTGGGTGGCACTGGCATTTAT	GTTTCTTCTGTTCCTCTTCGGAGA
Chkb exon 3-11	GCGTGATGTTCGCCATTCTC	GGAGAAACTTGAGGTGGGGTTG
Hoxa11	GGAGGAGAAGGAGCGACGG	TGGTATAAGGGCAGCGCTTTT
Hoxd11	AAGGCGGTGGCGGTGAAGGC	TCGCGGATCTGTACTTGGT
MMP9	ACGACATAGACGGCATCCA	GCTGTGGTTCAGTTGTGGTG
MMP13	GCCAGAACTTCCCAACCAT	TCAGAGCCCAGAATTTTCTCC
Cyclophilin	TCCAAAGACAGCAGAAAACTTTG	TCTTCTTGCTGGTCTTGCCATTCC

Table 2.1 Primer sequences used for genotyping and quantitative real-time PCR. Primer pair of 'Chkb exon 5-9' and 'Chkb exon 3-11' was used for genotyping. The remaining primer pairs were used for quantitative real-time PCR.

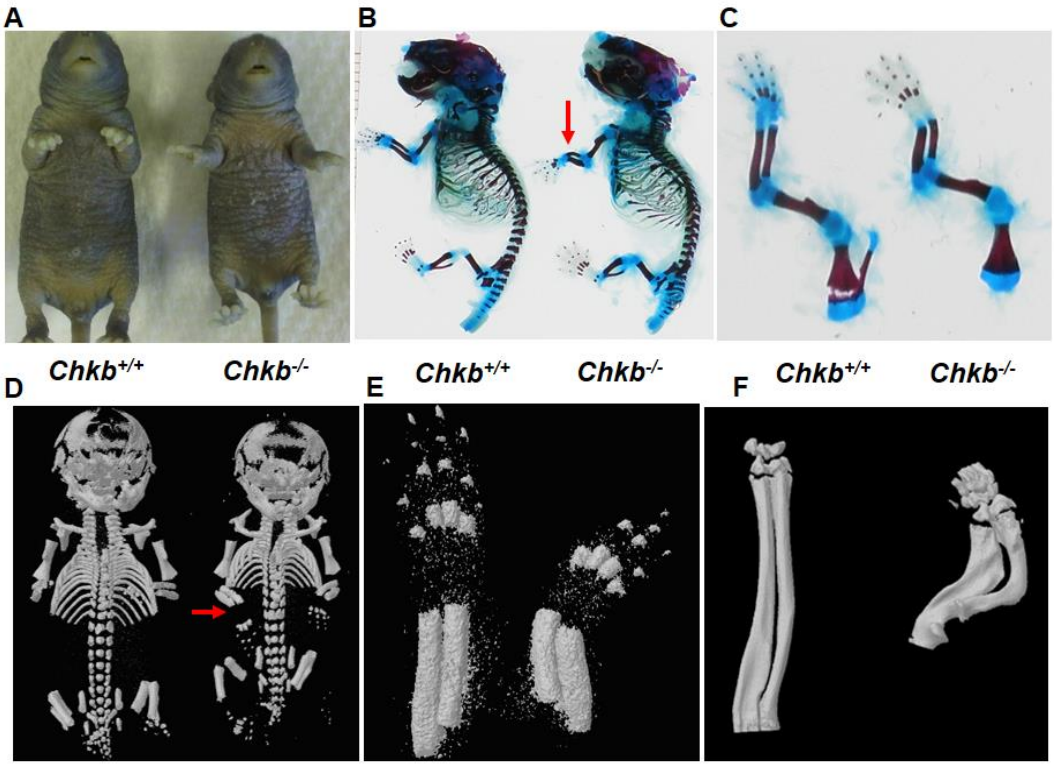

Figure 2.1 Deformation of the radius and ulna in *Chkb*$^{-/-}$ mice. Outward rational forelimb bone deformation was examined in 7-day-old *Chkb*$^{+/+}$ and *Chkb*$^{-/-}$ mice (A). Neonatal radius and ulna deformations were visualized by skeletal staining of whole body (B) and forelimbs (C) with Alizarin Red and Alcian Blue. Micro-CT imaging was performed on whole body (D) and forelimbs (E). Severe deformation of the radius and ulna in 30-day-old *Chkb*$^{-/-}$ mice was visualized by Micro-CT imaging (F). Red arrows indicate deformed radius and ulna in (B) and (D).

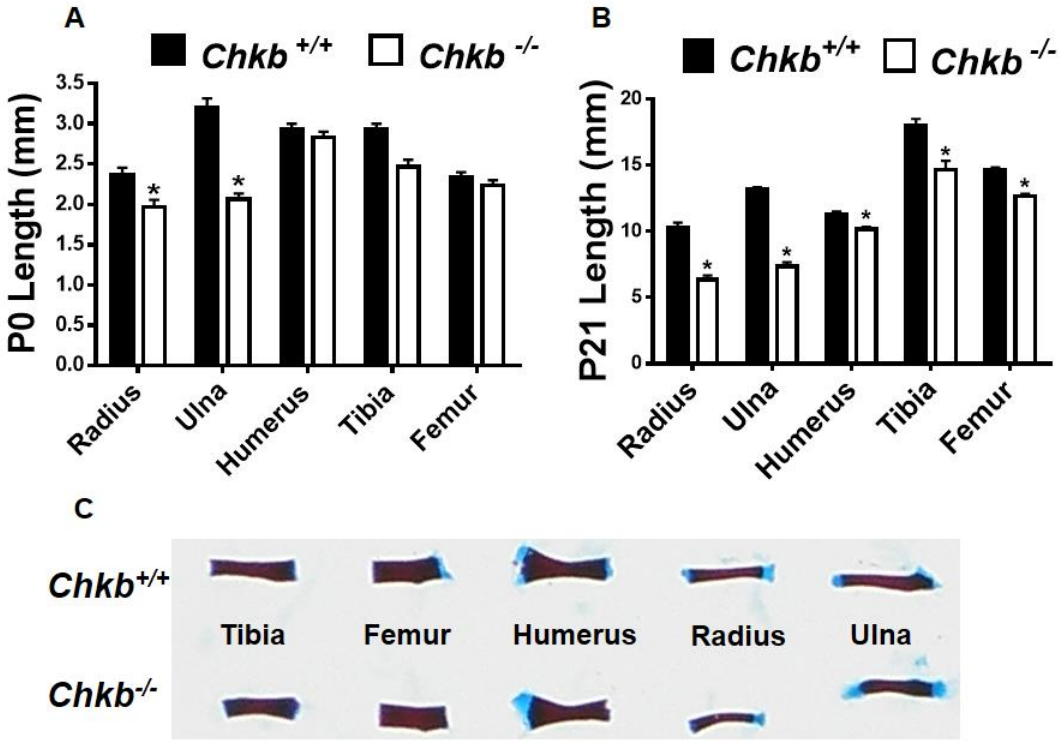

Figure 2.2 Lengths of radius and ulna are reduced. Long bones from newborn (P0) and 21-day-old (P21) mice were dissected and the bone lengths were measured (A, B). Data in (A) and (B) are means ± SEM from 4 littermates of each genotype; *: $P < 0.05$ compared to wild-type. Long bones from P0 mice were also stained with Alizarin Red and Alcian Blue (C).

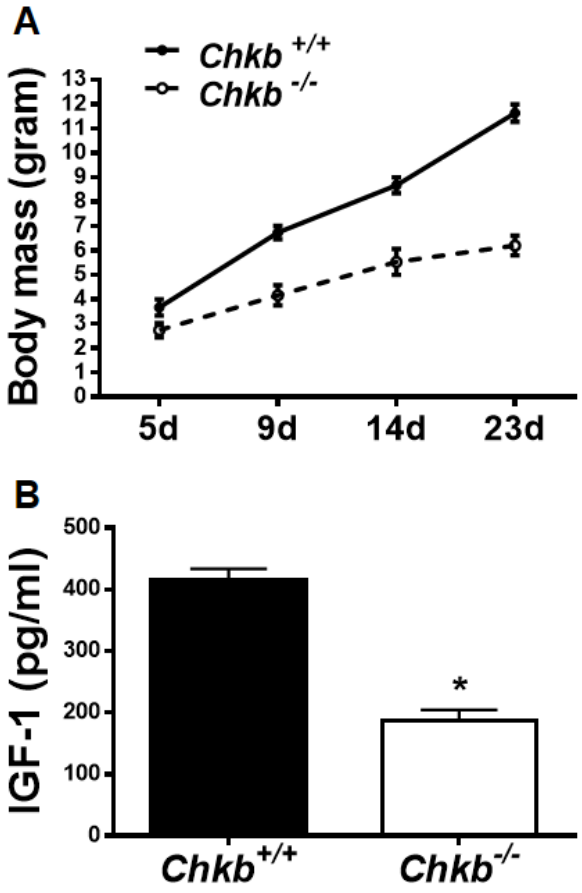

Figure 2.3 Growth retardation in *Chkb*$^{-/-}$ mice. Weight gain in *Chkb*$^{+/+}$ and *Chkb*$^{-/-}$ mice before weaning (A). Plasma concentration of insulin-like growth factor 1 (IGF-1) in 21-day-old *Chkb*$^{-/-}$ and *Chkb*$^{+/+}$ mice (B). Data are means ± SEM from 3-4 mice of each genotype; *: $P < 0.05$ compared to wild-type.

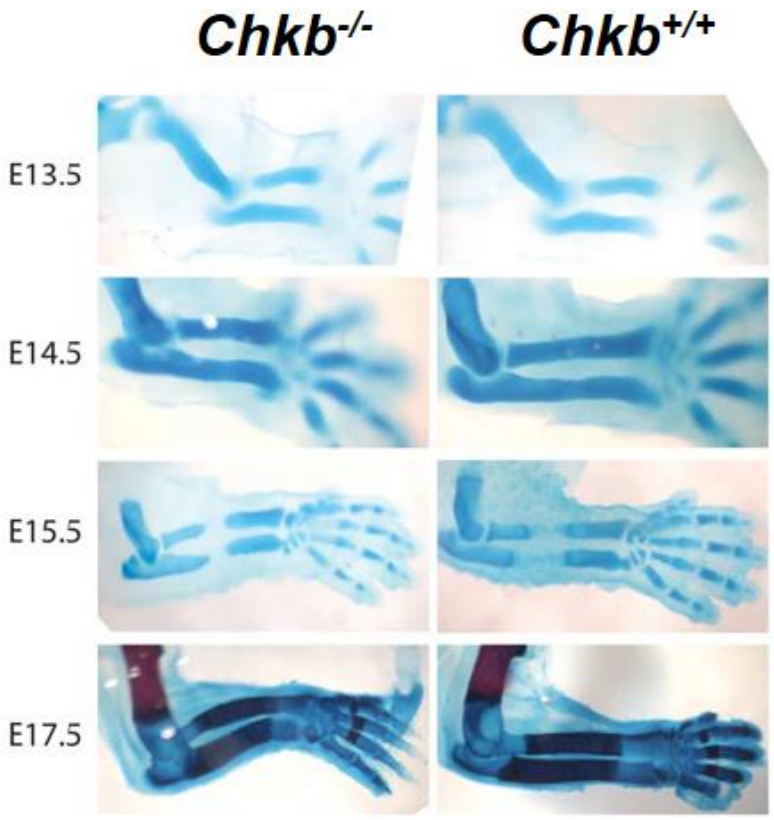

Figure 2.4 Deformations of the radius and ulna in *Chkb*$^{-/-}$ mice occur during late embryonic stage. Embryonic forelimbs were dissected free from soft tissues. E13.5-E15.5 forelimbs were then stained with Alcian Blue only whereas E17.5 forelimbs were stained with both Alizarin Red and Alcian Blue. E: embryonic day.

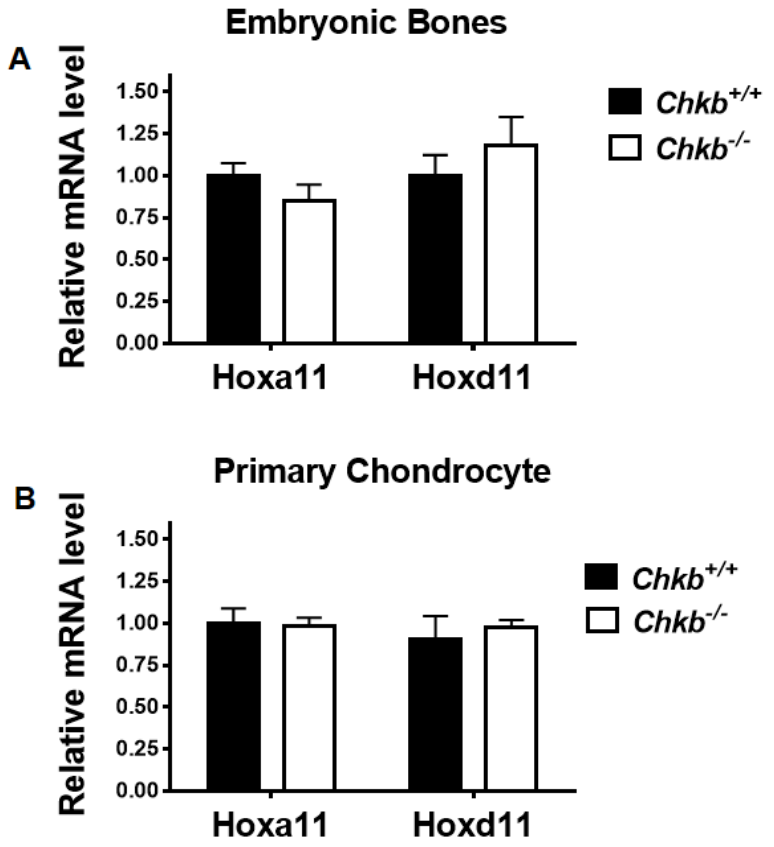

Figure 2.5 Expression of *Hoxa11* and *Hoxd11* genes in *Chkb*$^{+/+}$ and *Chkb*$^{-/-}$ mice. mRNA levels of Hoxa11 and Hoxd11 were analyzed in the radius and ulna of embryonic bone tissues (A) and E15.5 primary chondrocytes (B) of *Chkb*$^{-/-}$ and *Chkb*$^{+/+}$ mice by quantitative real-time PCR. Data are means ± SEM from 3 mice of each genotype; *: $P < 0.05$ compared to wild-type. E: embryonic day.

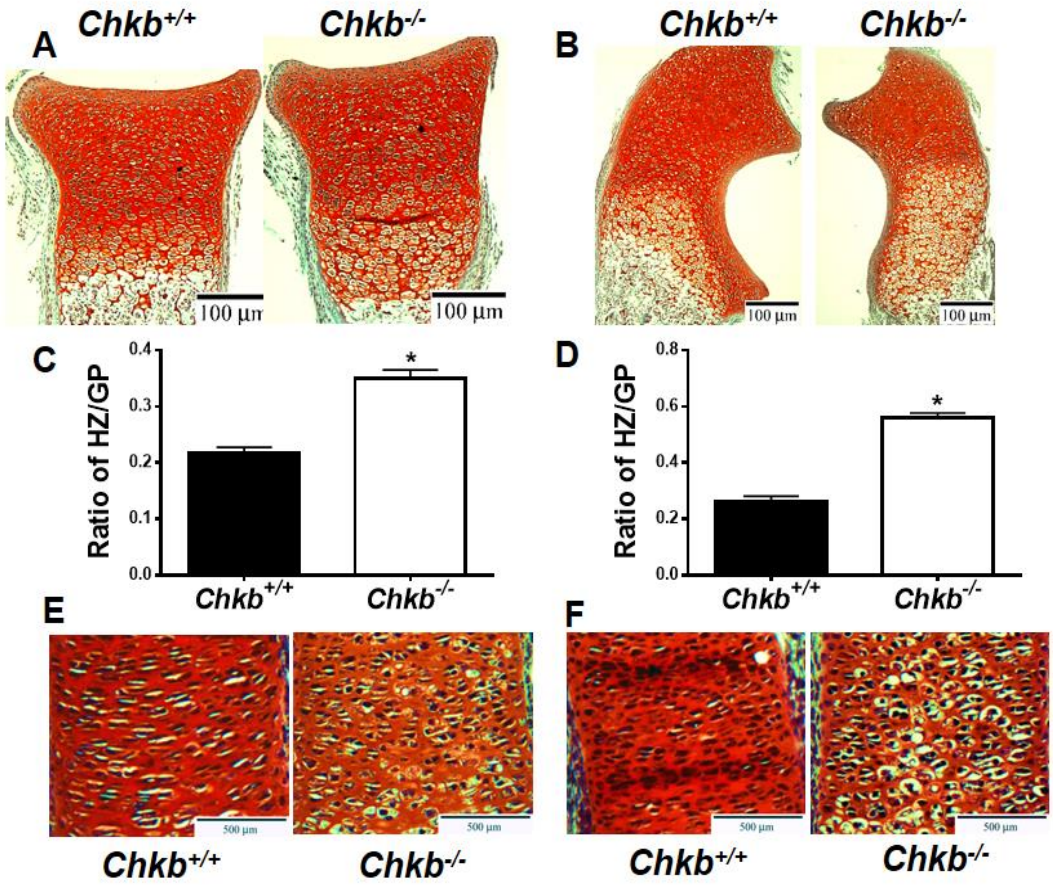

Figure 2.6 Growth plate defects in the radius and ulna of *Chkb*$^{-/-}$ mice. P0 (new born) radius (A), P0 ulna (B), E16.5 radius (E) and E16.5 ulna (F) were stained with Safranin O/fast green. The ratio of the length of hypertrophic zone (HZ) / the length of growth plate (GP) is shown in (C, D). Data in (C) and (D) are means ±SEM from 3 littermates of each genotype; *: $P < 0.05$ compared to wild-type. E: embryonic day.

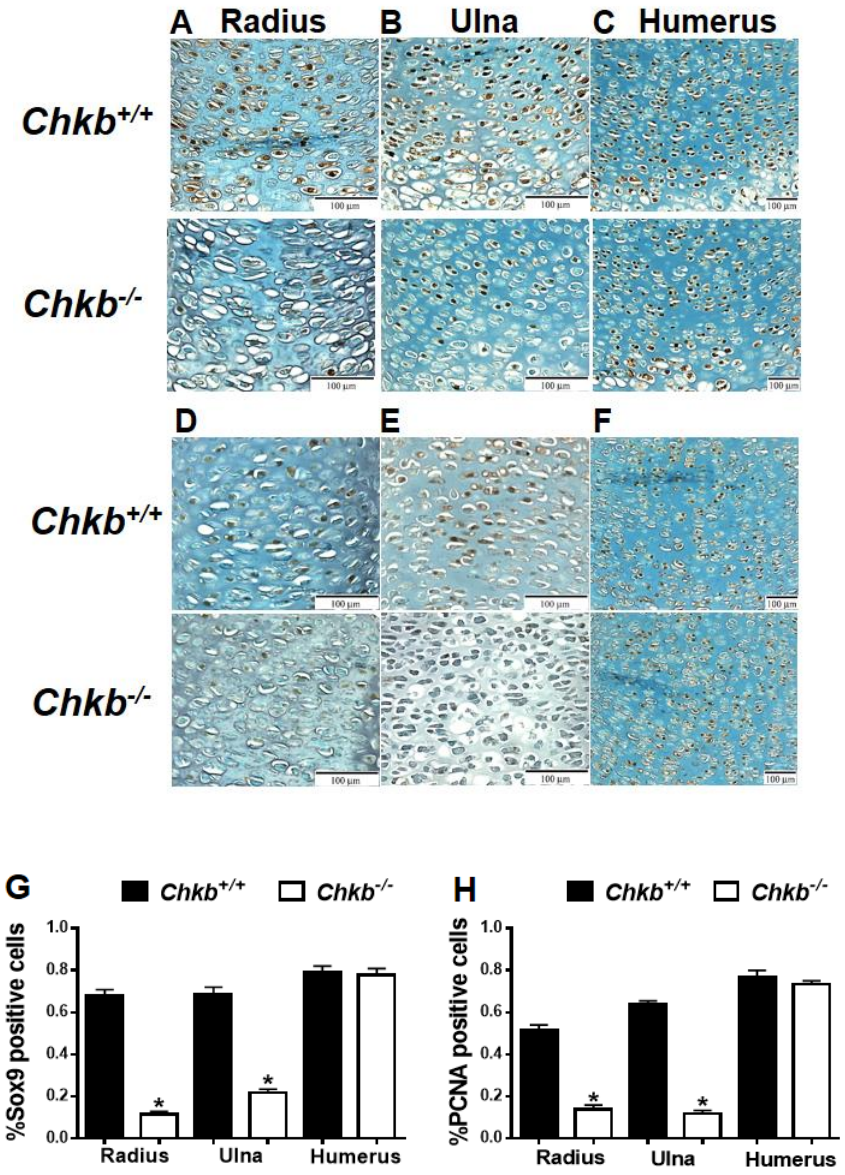

Figure 2.7 Chondrocyte differentiation and proliferation. Chondrocyte differentiation and proliferation were examined in E15.5 embryonic bone growth plates of the radius (A, D), ulna (B, E) and humerus (C, F) of *Chkb*$^{-/-}$ and *Chkb*$^{+/+}$ mice by Sox9 (A, B, C) and PCNA (D, E, F) immunohistochemistry. The percentage of positively stained cells was quantified (G, H). Data are means ± SEM from 3-4 littermates of each genotype; *: $P < 0.05$ compared to wild-type. E: embryonic day.

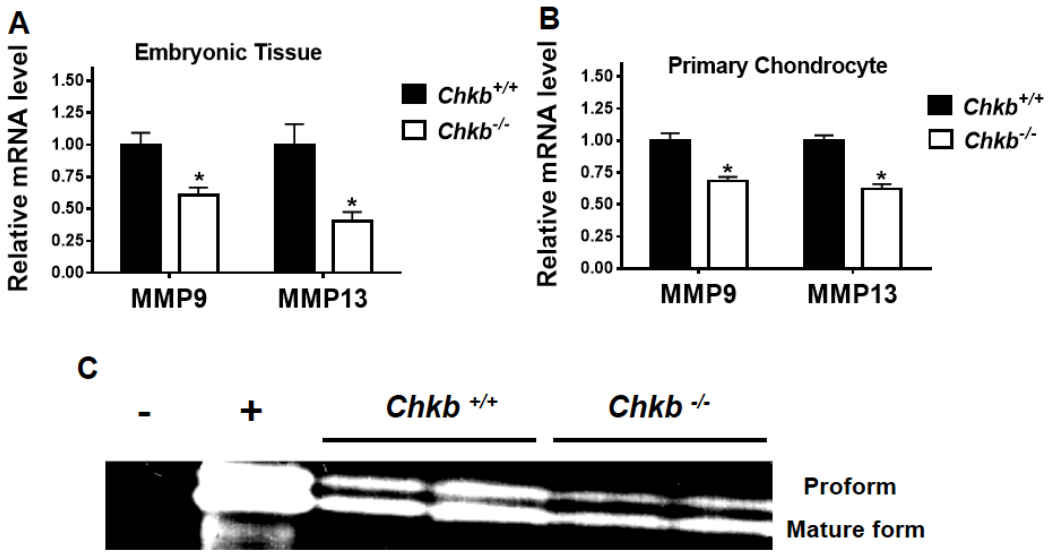

Figure 2.8 mRNA levels and activity of MMPs in the radius and ulna. mRNA levels of *Mmp9* and *Mmp13* were analyzed in the radius and ulna of embryonic bone tissues (A) and E15.5 primary chondrocytes (B) of $Chkb^{-/-}$ and $Chkb^{+/+}$ mice by quantitative real-time PCR. Data are means ± SEM from 3 littermates of each genotype; *: $P < 0.05$ compared to wild-type. (C): MMP9 activity was measured in primary chondrocytes from $Chkb^{-/-}$ and $Chkb^{+/+}$ mice. Gel shows the presence of the proform and the mature form in the gelatin zymography assay. - = distilled water (negative control); + = proteins from the chondrogenic cell line ATDC5 (positive control). E: embryonic day.

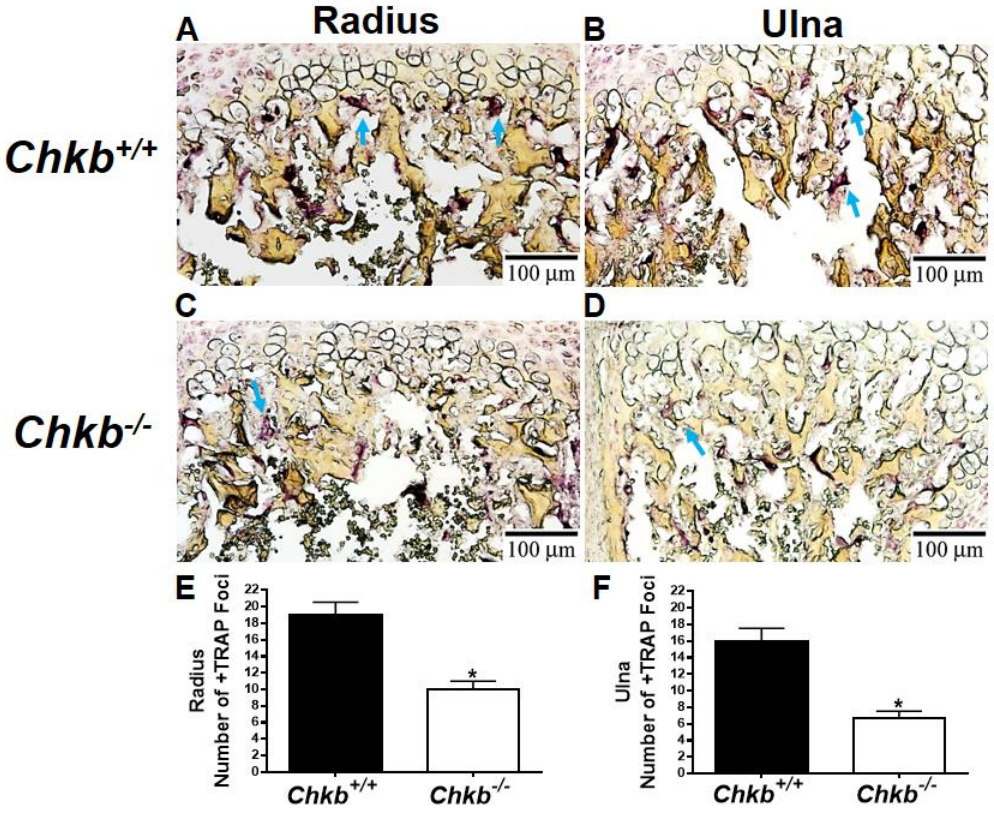

Figure 2.9 The radius and ulna of *Chkb*$^{-/-}$ mice contain fewer osteoclasts along the chondro-osseous junction. Bone sections from the radius (A, C) and ulna (B, D) of 7-day-old *Chkb*$^{+/+}$ (A and B) and *Chkb*$^{-/-}$ (C and D) mice were stained for the osteoclast marker, tartrate-resistant acid phosphatase (TRAP). Arrows show dark purple TRAP-positive cells. The number of TRAP-positive foci was quantified along the cartilage/bone interface (E, F). Data are means ± SEM from 3 littermates of each genotype; *: $P < 0.05$ compared to wild-type.

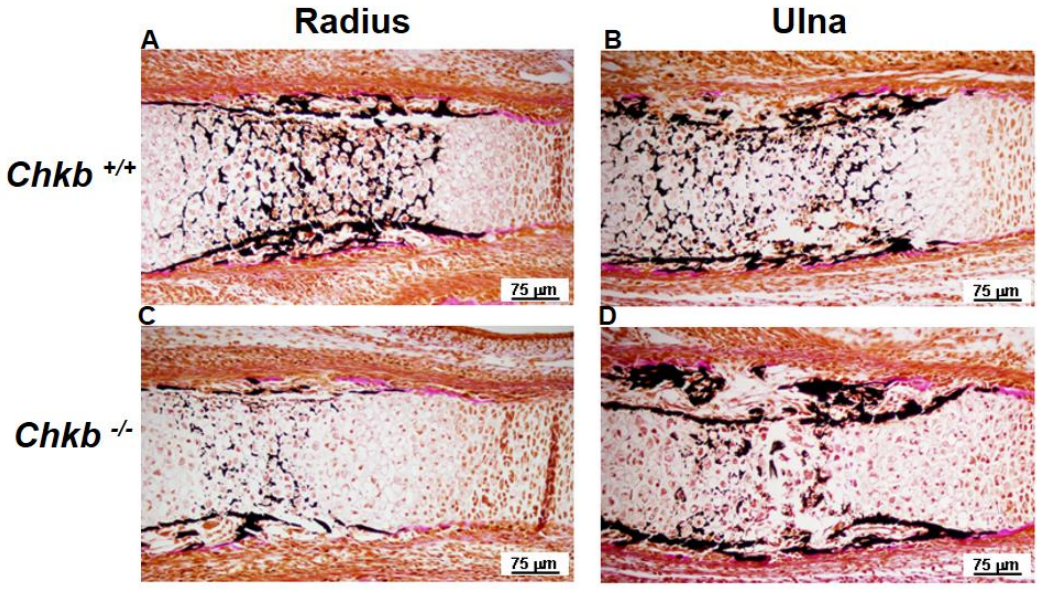

Figure 2.10 Formation of the primary ossification center in the radius and ulna. To evaluate formation of the primary ossification center, radius bones (A, C) and ulna bones (B, D) from E15.5 *Chkb*$^{+/+}$ (A, B) and *Chkb*$^{-/-}$ (C, D) mice were stained by the von Kossa method. Dark stain indicates mineralized bone. E: embryonic day.

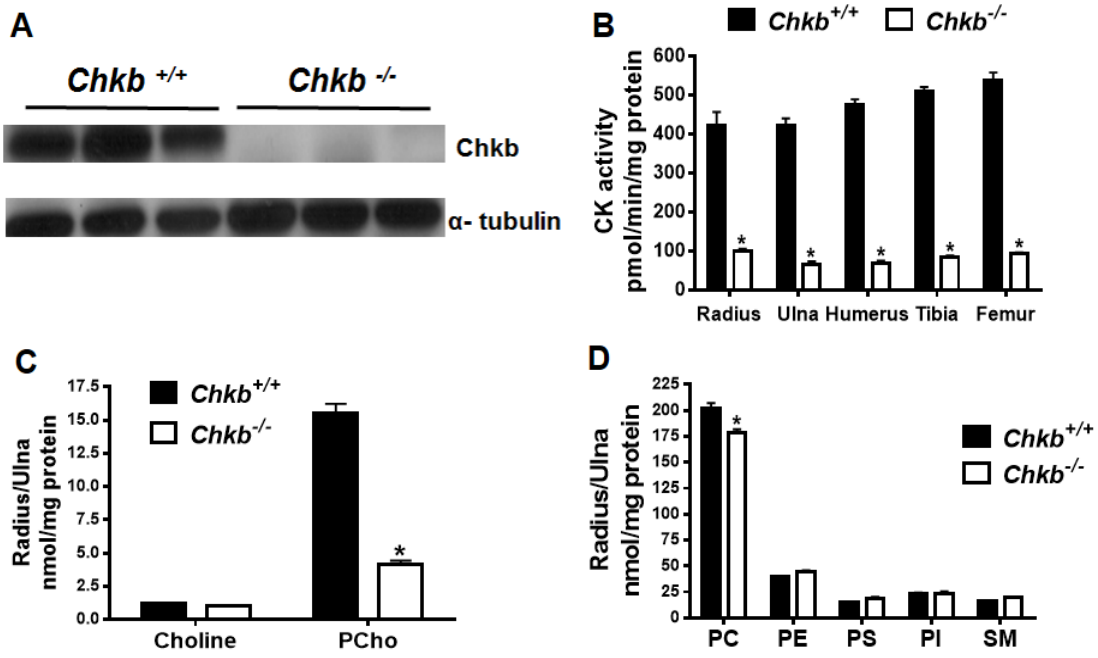

Figure 2.11 Biochemical analyses of primary chondrocytes. E15.5 radius and ulna were dissected free of soft tissue and primary chondrocytes were isolated for immunoblotting of CKβ (A), CK enzymatic activity (B), and quantification of choline metabolites (C) and phospholipids (D) by mass spectrometry. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin. Data in B-D are means ± SEM from 3 littermates of each genotype; *: $P < 0.05$ compared to wild-type. E: embryonic day.

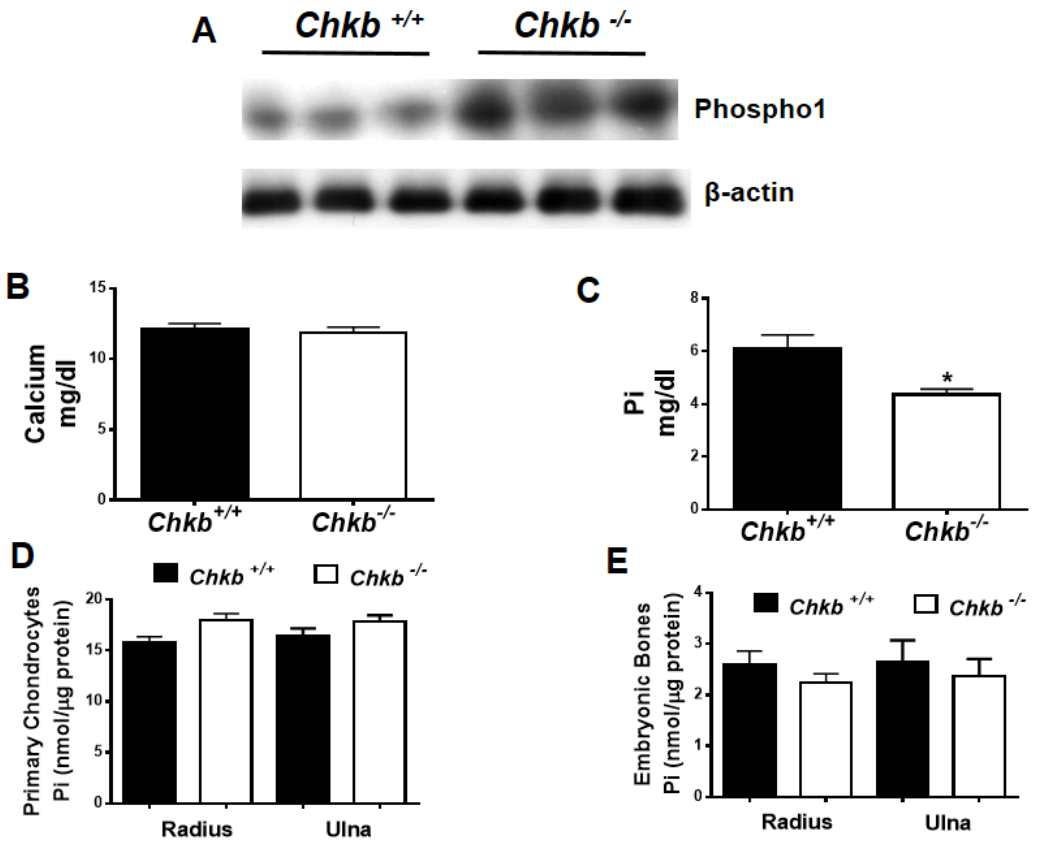

Figure 2.12 Chondrocyte mineralization in *Chkb*$^{-/-}$ and wild-type mice. Primary chondrocytes isolated from E15.5 embryonic radius and ulna were used to examine Phospho1 expression by immunoblotting with β-actin as a loading control (A). The serum from 21-day-old mice was used to measure calcium (B) and inorganic phosphate (Pi) (C) in *Chkb*$^{-/-}$ and *Chkb*$^{+/+}$ mice. Data are means ± SEM from 4 mice of each genotype; *: $P < 0.05$ compared to wild-type. Pi levels were also measured in E15.5 primary chondrocytes (D) and in radius and ulna bones (E) of *Chkb*$^{-/-}$ mice and wild-type mice. Data are means ± SEM from 3 mice of each genotype; *: $P < 0.05$ compared to wild-type. E: embryonic day.

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Chapter 3

Phosphatidylcholine Metabolism and Choline Kinase in Human Osteoblasts

3.1 Introduction

Choline kinase (CK) is the first enzyme in the CDP-choline pathway for the biosynthesis of phosphatidylcholine (PC), the most abundant phospholipid in mammalian cellular membranes (1, 2). CK is a cytosolic enzyme that catalyzes the conversion of choline to phosphocholine (PCho) (3). In mammals including humans, CK is encoded by two distinct genes, *Chkα* and *Chkb,* that express three isoforms CKα1, CKα2 and CKβ that are active as homodimeric or heterodimeric species (4). CKα1 and CKα2 differ only by the presence of an 18-residue insert in CKα2 (4). PC can be also synthesized through the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway, which primarily operates in the liver via sequential methylation reactions of phosphatidylethanolamine (PE) (5, 6). PE is the second most abundant phospholipid within mammalian membranes and is synthesized from both the CDP-ethanolamine pathway and the decarboxylation of phosphatidylserine (1, 7).

Although CK is not generally regarded as an important regulatory enzyme in the CDP-choline pathway, many studies have demonstrated that CK and its product, PCho, play essential roles in cell proliferation, transformation and human carcinogenesis (8-11). Recent studies also showed that CK is a crucial player in embryogenesis and normal functions of skeletal muscle (12-15). Deficiency of choline, the substrate of CK, is linked to abnormal bone development in turkeys and impaired bone remodeling in rats (16-18). Similarly, we found that mice lacking CKβ developed forelimb bone deformity (13), which indicates a potential involvement of CK in the formation/development of bone. Interestingly, two very recent human genetic studies showed that homozygous mutations in the gene coding for CTP: phosphocholine cytidylyltransferase (CT), the regulatory enzyme in the CDP-choline pathway to synthesize PC (19, 20), are identified in patients with bone disease, suggesting the importance of normal PC metabolism for skeletal development (21, 22).

Osteoblasts are cells that are responsible for the synthesis and mineralization of bone during both initial bone formation and later bone remodeling. In this study, we characterized CK and PC metabolism in two commonly used human osteoblast cells, primary human osteoblasts (HOB) and human osteosarcoma MG-63 cells. We found that in these human osteoblasts the CDP-choline pathway is the only route for PC biosynthesis and that CK does not catalyze the rate-limiting step for PC biosynthesis. However, CK activity and PCho are closely connected to the proliferation and mineralization of MG-63 cells.

3.2 Experimental Procedures

3.2.1 Cell culture

The human osteosarcoma cell line MG-63 was purchased from the American Type Culture Collection and the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum (FBS) (Gibco) at 37 ℃ in a humidified atmosphere, containing 5% CO_2. Primary human osteoblasts (HOB) were obtained from PromoCell (Heidelberg, Germany) and cultured in serum-free Osteoblast Basal Medium or Osteoblast Growth Medium containing 10% fetal calf serum (PromoCell) at 37 ℃ in a humidified atmosphere containing 5% CO_2.

3.2.2 Lipid extraction and separation of phospholipids and water-soluble choline metabolites

Osteoblast cells were harvested and lysed in buffer containing 10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor (1:100 dilution, Sigma). Cell lysates were pelleted by centrifugation at 1,000 rpm for 5 min and the supernatants were collected and sonicated. Protein was quantified by the Bradford procedure (23) and total lipids were extracted by the method of Folch et al (24). The organic phase containing the phospholipids was dried under nitrogen gas. After re-suspension in chloroform, phospholipids were separated by thin-layer chromatography in the solvent system chloroform/methanol/acetic acid/water 25:15:4:2 (v: v: v: v). The aqueous phase containing water-soluble choline metabolites was dried under nitrogen gas. The residues were re-suspended in methanol: water (1:1) and then separated by thin-layer chromatography in the solvent system methanol/0.5% sodium chloride/ammonia 10:10:1 (v: v: v). PC, PE, choline, PCho and CDP-choline were identified by

comparison to authentic standards (Avanti Polar Lipids) and radioactivity was measured by liquid scintillation counting.

3.2.3 Metabolic radiolabeling of cells

Osteoblast cells (~80% confluence) were washed twice at room temperature using DMEM or Osteoblast Basal Medium. [*methyl*-^{3}H]Choline (15 μCi) or [^{3}H]ethanolamine (15 μCi) (Amersham Biosciences) in 5 ml of DMEM or Osteoblast Basal Medium was added to cells in 100 mm culture plates and incubated for 30 min after which radioactive medium was removed and cells were incubated for an additional 2 h in corresponding medium lacking radioactivity. The cells were harvested in tissue homogenate buffer followed by lipid extraction as described above. The incorporation of [^{3}H]choline into choline metabolites and the incorporation of [^{3}H]ethanolamine into PE and PC were analyzed after isolation of the metabolites by thin-layer chromatography. [^{3}H]PC-labeled low density lipoproteins (LDL) and high density lipoproteins (HDL) were prepared as previously described (25-28). HOB and MG-63 cells were first incubated in serum-free medium overnight, and then incubated with 100 μg protein associated with 0.045μCi [^{3}H] of each kind of radiolabeled lipoproteins for 6 h before harvesting.

3.2.4 Immunoblot analyses

Osteoblasts were harvested in tissue homogenate buffer as described above. The homogenates were centrifuged at 348,000 × *g* for 15 min at 4 ℃ to obtain the cytosolic fraction. Proteins (100 μg) from either the cytosolic fraction or the whole cell lysate were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS, and immunoblotted with the relevant antibodies. The following antibodies were used: rabbit polyclonal anti-human CKα (1:200, Abcam); rabbit polyclonal anti-human CKβ (1:500, Sigma); rabbit polyclonal anti-human CyclinD1 (1:500, Santa Cruz Biotechnology); rabbit polyclonal anti-human proliferating cell nuclear antigen (PCNA) (1: 200, Abcam). Antibodies

raised against either β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to detect loading controls (Abcam). Densitometry of the immunoblots was analyzed by ImageJ 1.45s software (Wayne Rasband, NIH).

3.2.5 Choline kinase assay

Total CK activity was determined in the cytosolic fraction as previously described with minor modifications (29). The cytosolic fractions were incubated in a final volume of 100 μl buffer that contained 0.1 M Tris-HCl (pH 8.8), 10 mM ATP, 15 mM $MgCl_2$ and 0.25 mM [^{3}H]choline chloride (10.5 μCi/ml) at 37 ℃ for 30 min. The product PCho was separated using an AG1-X8 (200-400 mesh, OH-form) column (Bio-Rad). To determine the activity of each CK isoform, supernatant fractions were incubated with antisera (a gift from Dr. K. Ishidate, Teikyo Heisei University, Japan) raised against glutathione *S*-transferase (GST) (control), GST-CKα or GST-CKβ fusion proteins. Subsequently, protein A-Sepharose was added and the samples were incubated overnight at 4 ℃. The supernatant was used for CK assays. Activity not precipitated by CKα-specific antisera was due to β/β homodimers; activity not precipitated by CKβ-specific antisera was due to α/α homodimers; the remaining activity was due to α/β heterodimers (30).

3.2.6 Gene silencing by RNA interference

Stealth RNAi™ siRNA specific for *Chkα,* and negative control siRNA, were purchased from Invitrogen. Approximately 100 nmol/L of siRNA was transfected into MG-63 cells using Lipofectamine 2000 and Opti-MEM (Invitrogen).

3.2.7 RNA isolation and quantitative real-time PCR

Total RNA was isolated using TRIzol reagent, then reverse-transcribed by oligo (dT) and Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using a Rotor-Gene 3000 instrument and data were

analyzed using the Rotor-Gene 6.0.19 software (Montreal Biotech). Cyclophilin mRNA was used to normalize gene expression. The primers were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Primer sequences for *Chkα* were: forward primer, 5'-CATCTATTGAATTTGGGTACATGG-3'; reverse primer, 5'-GTCACACCCCAAGCTTCCT-3'. Primer sequences for *cyclophilin* were: forward primer, 5'-TCCAAAGACAGCAGAAAACTTTCG-3'; reverse primer, 5'TCTTCTTGCTGGTCTTGCCATTCC-3'.

3.2.8 Mass spectrometric analysis of lipids and water-soluble choline metabolites

Cell lysates (100 μg protein) from MG-63 cells were used for lipid extraction. The lipids were extracted according to the method described in (24) with internal phospholipid standards for quantification of the lipid species added at the stage of extraction. The lipids were quantified using liquid chromatography-mass spectrometry (LC-MS) as previously described (31). The acyl residues of the lipid species were determined using fragmentation analysis (32). The water-soluble choline metabolites were analyzed by LC-MS/MS as described previously (33).

3.2.9 Cell growth and proliferation assay

MG-63 cells were plated at a density of 1×10^5 cells/well in 6-well plates. Cells were incubated with control-siRNA or *Chkα*-siRNA at 37 ℃ for 24, 48 or 72 h. At the indicated times, culture medium was removed, the adherent cells were harvested with trypsin, and viable cells that excluded Trypan blue were counted using a hemocytometer.

3.2.10 Assay of mineralization capacity

Mineralization was induced by culturing confluent MG-63 cells in osteogenic growth medium containing 50 μg/ml ascorbic acid (Sigma) and 7.5 mM β-glycerophosphate (Sigma), supplemented with or without 50 μM

hemicholinium-3 (HC-3) (Sigma). The formation of mineralized nodules was detected at day 14 using Alizarin Red staining (34, 35). Briefly, the cells were rinsed twice with phosphate-buffered saline followed by fixation with 70% ethanol for 1 h at room temperature. The cells were then stained with 40 mM Alizarin Red (pH 4.2) for 30 min at room temperature, followed by three washes with water. The bound stain was then eluted with 10% cetylpyridinium chloride and the absorbance was measured at 550 nm.

3.2.11 Alkaline phosphatase (ALP) activity assay

MG-63 cells were washed three times with ice cold phosphate-buffered saline and scraped into freshly prepared collection buffer (10 mM Tris-HCl; 0.1% Triton X-100). ALP activity was measured in cell extracts at 37 ℃ for 30 min using liquid *p*-nitrophenyl phosphate as substrate (Sigma). The colorimetric determination of the product (*p*-nitrophenol) was performed at 405 nm and normalized to the amount of protein.

3.2.12 Statistical analysis

All statistical analyses were performed using GraphPad Prism software 6.0. Graphs show mean values ± standard error of the mean (SEM) of three independent experiments. All statistical tests were two-sided and were considered to be statistically significant at $P < 0.05$.

3.3 Results

3.3.1 The incorporation of [^{3}H]choline and [^{3}H]ethanolamine into PC in human osteoblasts

We studied the biosynthesis of PC in two commonly used human osteoblast models: primary human osteoblasts (HOB) and human osteosarcoma MG-63 cells. Cells were pulse-labeled with [^{3}H]choline chloride for 30 min followed by a 2 h chase without radioactivity. The incorporation of radiolabeled choline into PC was rapid and robust in both types of osteoblast cells; more radioactivity/mg protein was incorporated into PC in MG-63 cells than in HOB cells (Figure 3.1A).

To evaluate the presence of the PEMT pathway in human osteoblasts, both HOB and MG-63 cells were labeled with [^{3}H]ethanolamine. The incorporation of radiolabeled ethanolamine into PE was rapid and significant, whereas the radioactivity recovered in PC was barely detectable (Figure 3.1B), indicating that the PE methylation pathway is not a functional route for PC biosynthesis in human osteoblast cells.

3.3.2 The incorporation of lipoprotein-derived PC into human osteoblasts

In recent years it has been demonstrated that in addition to liver, bone is also a quantitatively important organ for the uptake of circulating lipoproteins (36-39). Consequently, we determined if plasma lipoproteins could also deliver PC to osteoblasts. Freshly isolated [^{3}H-PC] LDL or [^{3}H-PC] HDL was added to the osteoblasts for 6 h after which the cells were harvested and the incorporation of radioactivity into the PC fraction was determined. Significant amounts of radioactivity from LDL and HDL were detected in cellular PC in both types of osteoblast cells (Figure 3.2). Thus, as in cultured primary hepatocytes (25-28), lipoproteins can be a source of osteoblast PC.

3.3.3 PC biosynthesis in human osteoblasts

PC biosynthesis in the osteoblasts was assessed by measuring the incorporation of radioactive choline into PC via the CDP-choline pathway. HOB and MG-63 cells were pulse-labeled with [^{3}H]choline for 30 min followed by a 1 or 2 h chase without radioactivity. The incorporation of radioactivity into choline, PCho, CDP-choline and PC was measured. Figure 3.3 shows a rapid loss of radioactivity from choline and its appearance into PCho. As the accumulated radioactivity in PCho decreased, the [^{3}H] was quantitatively recovered in PC. Thus, PC is synthesized from choline in HOB and MG-63 cells. In addition, these data strongly imply that the rate-limiting step for PC biosynthesis in these osteoblast cells is catalyzed by CTP: phosphocholine cytidylyltransferase (CT), the enzyme that converts PCho to CDP-choline.

3.3.4 Choline kinase activity and amounts of CK protein in human osteoblasts

We next measured CK activity, as well as the amounts of CKα and CKβ proteins. The total CK activity in MG-63 cells was approximately double that in HOB cells (Figure 3.4A), consistent with the greater level of CKα protein in MG-63 cells compared to HOB cells (Figure 3.4B). In contrast, the amount of CKβ protein was equivalent in the two types of osteoblast cells. The amount of PCho was also higher in MG-63 cells than in HOB cells (Figure 3.4C). Thus, compared to HOB cells, MG-63 cells contain higher CK activity, higher CKα protein expression and a greater amount of PCho.

3.3.5 The contribution of choline kinase isoforms to total CK activity in human osteoblasts

The contribution of CKα and CKβ to total CK activity was also analyzed in the osteoblasts. CKα or CKβ antisera were used to sequester the corresponding isoform to display the contribution from CKβ or CKα, respectively. CKα accounted for ~ 50% of total CK activity in HOB cells, whereas ~ 80% of total

CK activity in MG-63 cells was due to CKα (Figure 3.4D). These data indicate that CKα is the dominant CK isoform in MG-63 cells whereas CKα and CKβ contribute approximately equally to total CK activity in HOB cells.

3.3.6 *Chkα* gene silencing in MG-63 cells

Since CKα is the major contributor of total CK activity in MG-63 cells, we investigated the possible role of CKα in PC biosynthesis and osteoblast functions in these cells. *Chkα* gene silencing was performed in MG-63 cells using siRNA. After incubation of the cells with *Chkα*-siRNA for 72 h, the amount of CKα mRNA was decreased by approximately 80% (Figure 3.5A) and the amount of CKα protein was similarly reduced (Figure 3.5B) compared to cells treated with control-siRNA. Silencing of *Chkα* did not affect expression of the non-targeted CKβ isoform, indicating that compensatory mechanisms were not induced (Figure 3.5B). Consistent with the decrease in CKα expression, CK activity was reduced by ~75% in MG-63 cells treated with *Chkα*-siRNA (Figure 3.5C).

3.3.7 *Chkα* gene silencing increases the amount of choline and decreases PCho but has no effect on cellular PC

To determine if *Chkα* gene silencing influenced the cellular levels of choline metabolites and phospholipids, MG-63 cells that had been treated with *Chkα*-siRNA were used for mass spectrometric quantification of these metabolites. Figures 3.6A and B show that choline levels approximately doubled whereas the amount of PCho decreased by ~ 80% after *Chkα* gene silencing. This reduction in PCho is consistent with the decrease in CKα expression and CK activity (Figure 3.5). However, attenuation of CKα expression did not reduce the cellular mass of PC or other major phospholipids (Figure 3.6C), indicating that the residual CK activity in the MG-63 cells in which CKα expression had been reduced was sufficient for maintaining a normal level of PC. These observations also support the idea that CK is not the rate-limiting enzyme for PC biosynthesis in MG-63 osteoblast cells.

3.3.8 *Chkα* gene silencing attenuates cell growth and proliferation

Many studies have demonstrated that CK plays an essential role in tumor cell growth and proliferation (8-11). To examine how *Chkα* gene silencing could affect the growth and proliferation of human osteoblasts, MG-63 cells were treated with siRNA for 24, 48 or 72 h. After each time interval, the cells were isolated by trypsinization and the total number of live cells was quantified. Although treatment of the cells for 24 h with *Chkα*-siRNA had no effect on the total number of cells, treatment for 48 and 72 h reduced the cell number by approximately 35% and 45%, respectively (Figure 3.7A). Thus, over time *Chkα*-siRNA treatment gradually inhibited cell growth. To obtain insight into the cellular mechanisms underlying this inhibition, we examined the expression of cell proliferation markers PCNA and CyclinD1 by immunoblotting. The expression of both PCNA and CyclinD1 was reduced by approximately 50% after *Chkα* gene silencing (Figures 3.7B, C), indicating attenuated cell proliferation. These data suggest that CKα plays an essential role in normal cell proliferation of MG-63 osteoblasts.

3.3.9 Inhibition of choline kinase decreases osteoblast mineralization capacity

Hemicholinium-3 (HC-3), a compound with a structure similar to that of choline, inhibits CK activity in many types of cells (40-42). In addition, X-ray crystallographic studies on CK isoforms complexed with HC-3 show that HC-3 is a more potent inhibitor of CKα than of CKβ (43). Since induction of mineralization occurs after approximately 14 days in MG-63 cells (34, 35), we used HC-3 instead of gene silencing to determine if inhibition of CK impaired osteoblast mineralization capacity. Incubation of MG-63 cells with 50 μM HC-3 reduced the total CK activity by ~80% (Figure 3.8A), which is comparable to the extent of inhibition induced by *Chkα*-siRNA treatment (Figure 3.5C). Mineralization was induced in MG-63 cells by incubation with osteogenic growth medium with or without 50 μM HC-3 for 14 days after which mineralization was measured by staining the cells with Alizarin Red. In cells treated with 50 μM HC-

3, mineralization was ~40% lower than in control cells (Figure 3.8B). We also measured the activity of alkaline phosphatase (ALP), the key enzyme that controls bone mineralization (44-46), in HC-3 treated cells. Inhibition of CK activity by HC-3 decreased ALP activity by ~30% (Figure 3.8C). Together, these data suggest that CK plays an important role in the mineralization process of human osteosarcoma MG-63 cells.

3.4 Discussion

Currently, knowledge of PC metabolism in human osteoblast cells is limited, as is the potential relationship between CK and the function of osteoblasts. In this study, we characterized PC metabolism in two human osteoblast models, primary human osteoblast cells (HOB) and human osteosarcoma MG-63 cells. We also compared the expression and activity of CK in HOB and MG-63 cells. By gene silencing of *Chkα* and inhibition of CK activity in MG-63 cells, we also demonstrated that CKα has a contributing role in the growth and mineralization of MG-63 cells.

3.4.1 Sources of PC in human osteoblast cells

PC can be synthesized by either the CDP-choline pathway or via the sequential methylation of PE; the latter route of PC synthesis is believed to be quantitatively significant only in liver (5, 6). Our studies demonstrate that [^{3}H]choline is rapidly incorporated into PC in both HOB and MG-63 cells (Figure 3.1A). The incorporation of [^{3}H]ethanolamine into PE was also rapid and robust, but the incorporation of [^{3}H]ethanolamine into PC via the methylation of PE was barely detectable (Figure 3.1B). This observation indicates that the PEMT pathway is not a significant source of PC in human osteoblasts, consistent with a previous study in which PC biosynthesis via PE methylation was not detected in mouse calvaria tissues (47). The involvement of lipoproteins in bone metabolism has been reported in recent years (36-39). Thus, we also considered that a source of PC for osteoblasts might be circulating lipoproteins. Indeed, the low density lipoprotein receptor related protein 1 (LRP1), the low density lipoprotein receptor (LDLR) and scavenger receptor B1 are expressed in osteoblasts (37, 39). Consistent with this literature, our experiments revealed that lipoproteins can deliver PC to human osteoblasts in both HOB and MG-63 cells (Figure 3.2).

3.4.2 PC biosynthesis in human osteoblast cells

To obtain more insights into PC biosynthesis via the CDP-choline pathway, we performed [^{3}H]choline radiolabeling experiments in both HOB and MG-63 cells. Radiolabeled choline was rapidly converted into PCho and radioactivity accumulated in PCho in both types of osteoblast cells (Figure 3.3). This finding suggests that CTP: phosphocholine cytidylyltransferase (CT), the enzyme that converts PCho to CDP-choline, is the rate-limiting enzyme for PC biosynthesis in human osteoblasts. Under most metabolic conditions, and in most cell types, CT is considered to be the limiting or regulatory enzyme in the CDP-choline pathway for PC biosynthesis (19, 20). In the radiolabeling experiments with [^{3}H]choline, MG-63 cells contained less [^{3}H]choline, but more [^{3}H]PCho, during the chase period than did HOB cells (Figure 3.3). This observation suggests that MG-63 cells convert choline into PCho more rapidly than do HOB cells. This conclusion was supported by the demonstration that CK activity is higher in MG-63 cells than in HOB cells (Figure 3.4A).

3.4.3 Characterization of choline kinase in human osteoblast cells

Our experiments demonstrated that MG-63 cells have ~2-fold higher total CK activity than do HOB cells (Figure 3.4A). These data are consistent with the finding that MG-63 cells contained almost twice as much PCho as did HOB cells (Figure 3.4C). We also showed that CKα is the dominant CK isoform in MG-63 cells but not in HOB cells (Figure 3.4D). Many studies have demonstrated that CKα and PCho play essential roles in human cell transformation and carcinogenesis (8-11). Moreover, increased CKα expression and CK activity, as well as higher PCho levels, have been detected in numerous human tumors and cancer cells (48-50). Thus, the amount of PCho is sometimes used as a marker of malignancy in the diagnosis and monitoring of progression of cancer (51-53). Therefore, the finding that CK activity, CKα expression and PCho levels in human osteosarcoma MG-63 cells were higher than in HOB cells was not

unexpected. This finding also suggests that, as in other types of tumor cells, CKα might represent a potential drug target for treatment of bone cancers.

3.4.4 Silencing of *Chkα* does not reduce the amount of PC but decreases intracellular PCho in MG-63 cells

We utilized mass spectrometry to determine the pool sizes of choline metabolites and phospholipids in MG-63 cells treated with *Chkα*-siRNA. Silencing of CKα expression by ~80% resulted in higher levels of choline, significantly reduced levels of PCho but unchanged amounts of cellular PC (Figure 3.6). These findings are consistent with previous studies in which choline levels were higher in *Chka*$^{+/-}$ mice than in wild-type mice whereas the amount of PCho was lower and the amount of PC was unaltered (12), in liver and testis, the two mammalian tissues in which CKα is most abundantly expressed (4). The amount of PCho was reduced by ~80% in the *Chkα*-siRNA-treated cells (Figure 3.6B), which correlated directly with the ~75% decrease of CK activity (Figure 3.5C). However, CKα knockdown did not affect cellular PC mass, further supporting the proposal that CK is not the limiting or regulatory enzyme in CDP-choline pathway of PC biosynthesis in MG-63 cells.

3.4.5 Silencing of *Chkα* decreases cell growth and proliferation in MG-63 cells

Silencing of *Chkα* in MG-63 cells gradually compromised cell growth (Figure 3.7A) and decreased the expression of PCNA, a commonly used marker of cell proliferation (Figures 3.7B, C). These results are consistent with previous findings which showed that knockdown of CKα in breast cancer cells decreased cell proliferation and tumor xenograft growth (54-56). We also observed a ~50% reduction in expression of CyclinD1 (Figures 3.7B, C), a key protein involved in cell cycle control that is required for G1/S phase transition (57). These observations suggest that silencing of *Chkα* decreases cell proliferation by impairing progression of the cell cycle. Our data are in excellent agreement with a previous microarray study in which over-expression of CKα in human HEK293T

cells up-regulated CyclinD1 expression, whereas inhibition of CKα down-regulated CyclinD1 expression (58). Moreover, the decrease in cell proliferation induced by silencing of CKα might be also due to the dramatic reduction of intracellular PCho (Figure 3.6B), since production of PCho has been suggested to be an important process in the induction of mitogenesis by growth factors in several types of human cells. In these types of human cells, inhibition of CK activity reduced intracellular PCho levels and blocked the growth factor-induced synthesis of DNA (59-61).

Phospholipid mass is doubled during cell division and PC biosynthesis has been shown to be a key determinant of normal cell cycle progression for cell proliferation (62, 63). However, in our study, the decreased cell proliferation was not accompanied by reduced PC mass after *Chkα*-siRNA treatment (Figure 3.6C). Thus, we believe that the observed attenuated cell growth resulted from decreased CK activity and lower PCho level, which lead to impaired cell cycle progression.

3.4.6 Inhibition of CK activity impairs mineralization capacity in MG-63 cells

Inhibition of CK activity by HC-3 decreased the mineralization capacity of MG-63 cells, probably due to the reduction of ALP activity (Figures 3.8B, C), the key phosphatase that generates inorganic phosphate (Pi) during mineralization process (44-46). However, no previous study has revealed any direct connection between CK and ALP and we have not obtained data that could implicate the potential relationship between them. Therefore, future study is required to investigate the mechanism in which CK activity inhibition leads to decreased ALP activity.

In addition to producing PCho, CK can also phosphorylate ethanolamine to phosphoethanolamine (PEA) (64, 65). A connection between CK and bone mineralization is that several recent studies have shown that PCho and PEA are specific substrates of Phospho1 to generate Pi (66, 67), a recently identified phosphatase that is expressed in mineralizing tissue (68-72). Thus, a speculation

could be that inhibition of CK activity would deplete the substrate availability for Phospho1 and limit its activity in the mineralization process. However, we found Phospho1 is not expressed in MG-63 cells compared to another human osteosarcoma SaOS-2 cells that have Phospho1 expression (Figure 3.9). These data are consistent with a previous study which also showed that Phospho1 expression is absent in MG-63 cells (68). Thus, Phospho1 does not play a role in the observed decreased mineralization activity induced by CK inhibition.

3.5 Conclusion

We have characterized PC metabolism in human osteoblasts and shown that CK, particularly CKα, contributes to the growth and mineralization of human osteosarcoma cells. To our knowledge, this is the first study that reveals a connection between CK and the function/growth of human osteosarcoma cells. The possibility that inhibition of CK might be a useful therapy for bone cancer should be considered.

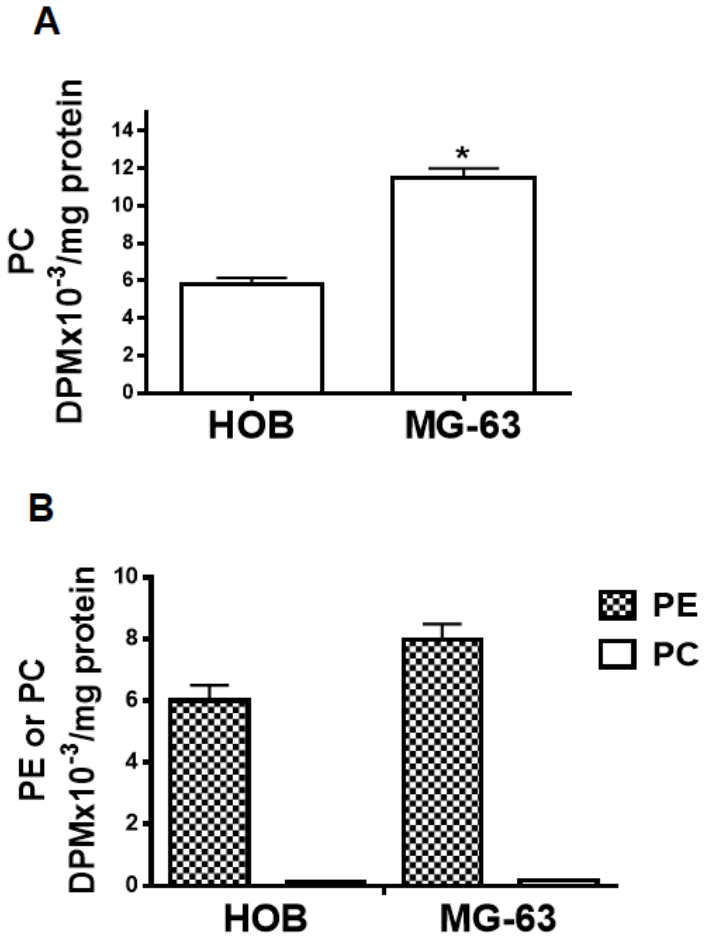

Figure 3.1 The incorporation of [^{3}H]choline and [^{3}H]ethanolamine into cellular phospholipids of human osteoblasts. MG-63 cells or HOB cells were pulse-labeled for 30 min with 15 μCi of [*methyl*-^{3}H]choline (A) or [^{3}H]ethanolamine (B) and subsequently incubated for up to 2 h in medium lacking radioactivity. At the end of the chase period, the cells were harvested and the amount of radioactivity was measured in PC and PE. Data are means ± SEM from 3 independent experiments. * $P < 0.05$.

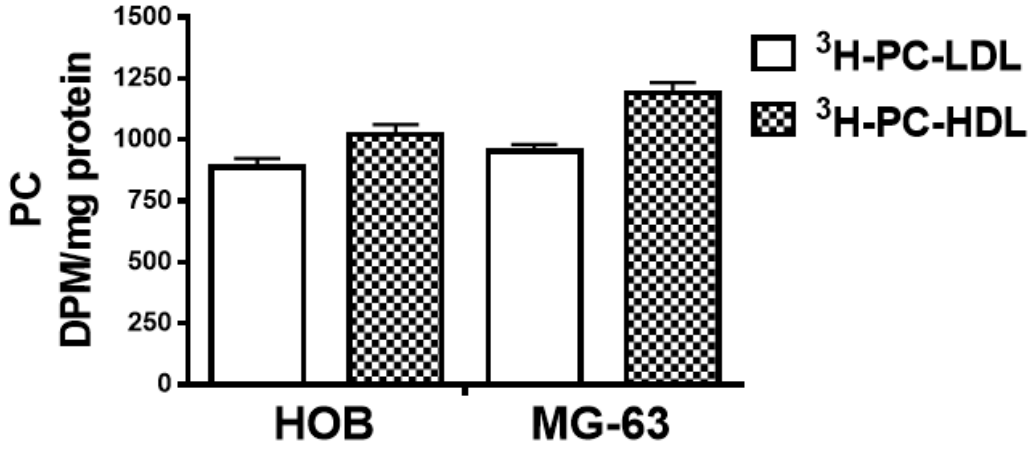

Figure 3.2 The incorporation of [^{3}H-PC]lipoproteins into cellular phosphatidylcholine of human osteoblasts. Low density lipoproteins (LDL) or high density lipoproteins (HDL) containing [^{3}H]PC were incubated for 6 h with HOB and MG-63 cells that had been deprived of serum overnight. The incorporation of lipoprotein-derived [^{3}H]PC into cellular PC was quantified.

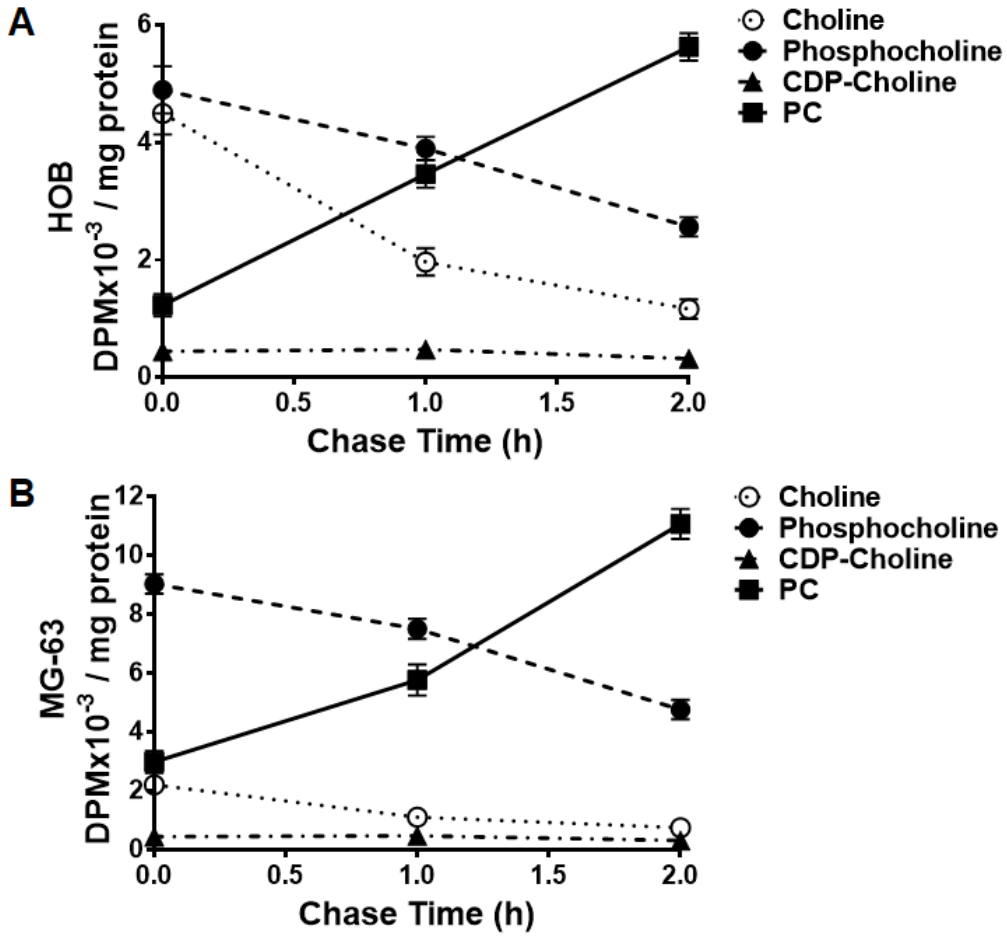

Figure 3.3 The incorporation of [^{3}H]choline into choline-containing compounds in human osteoblasts. HOB and MG-63 cells were pulse-labeled for 30 min with 15 μCi of [*methyl*-^{3}H]choline and subsequently incubated for up to 2 h in medium lacking radioactivity. At the end of the chase period, the cells were harvested and radioactivity was quantified in choline, PCho, CDP-choline and PC. Data are means ± SEM from 3 independent experiments.

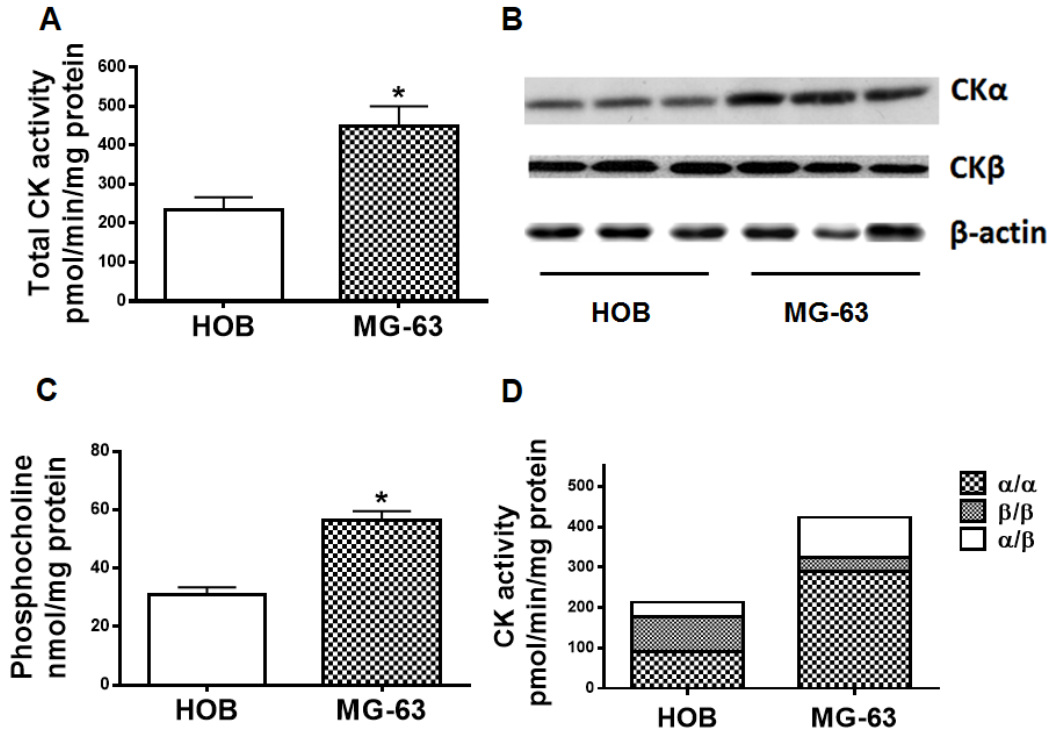

Figure 3.4 CK activity in MG-63 and HOB cells. Cytosol was prepared from cell lysates of MG-63 and HOB cells. CK activity (A) and protein expression of CKα and CKβ (B) were analyzed. β-actin was used as a loading control for CK expression. The amount of PCho in cell lysates was also measured (C). The contributions of CKα and CKβ isoforms to total CK activity were determined (D). Data are means ± SEM from 3 independent experiments. * $P < 0.05$.

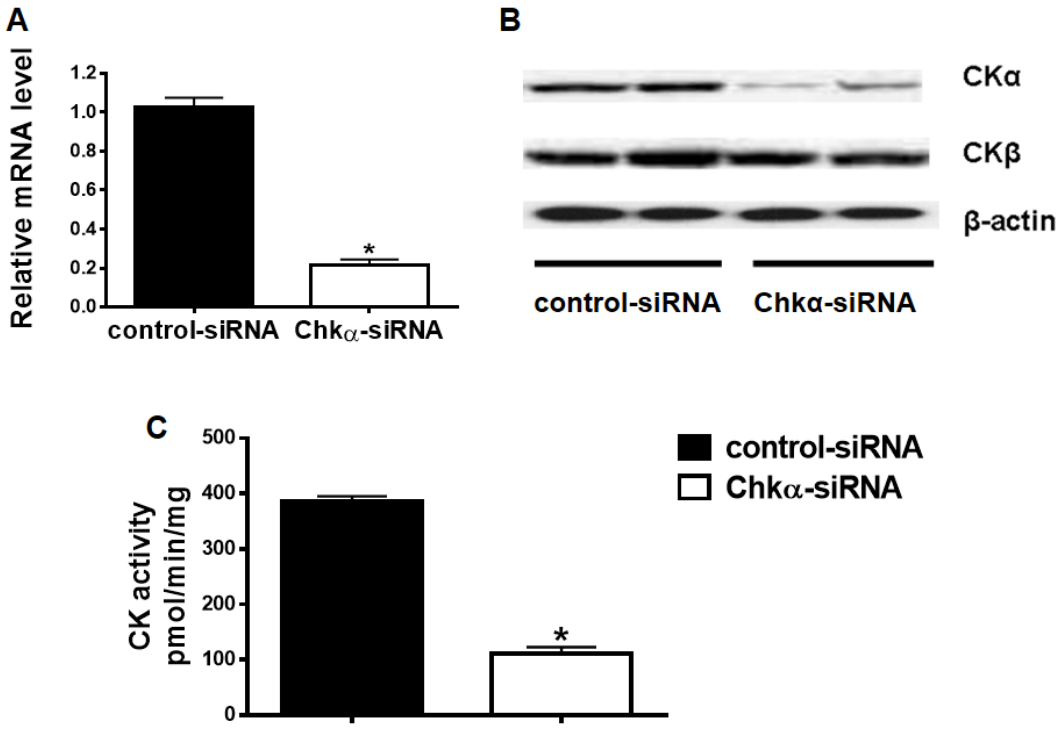

Figure 3.5 *Chkα* gene silencing in MG-63 cells. *Chkα* siRNA (100 nmol/L) was transfected into MG-63 cells for 72 h after which the level of CKα mRNA (A), the amounts of CKα and CKβ proteins (B) and total CK activity (C) were analyzed by quantitative real-time PCR, immunoblotting and CK enzymatic assay, respectively. The amount of β-actin was used as a loading control for the immunoblotting (B). Data are means ± SEM from 3 independent experiments. * $P < 0.05$.

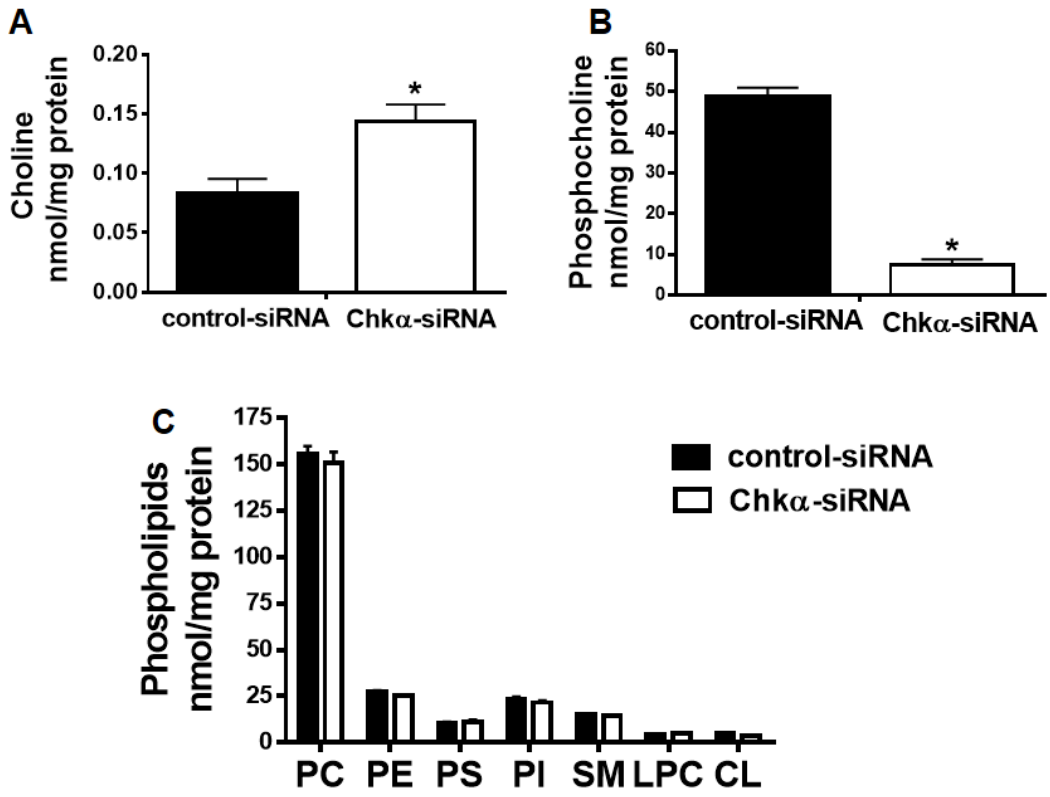

Figure 3.6 Amounts of choline metabolites in MG-63 cells in which expression of the *Chkα* gene was silenced. *Chkα*-siRNA (100 nmol/L) was transfected into MG-63 cells for 72 h after which the amounts of choline (A), PCho (B) and phospholipids (C) were measured in cell extracts (100 μg protein) by mass spectrometry. Data are means ± SEM from 3 independent experiments. **P* < 0.05.Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; LPC, lysoPC; CL, cardiolipin.

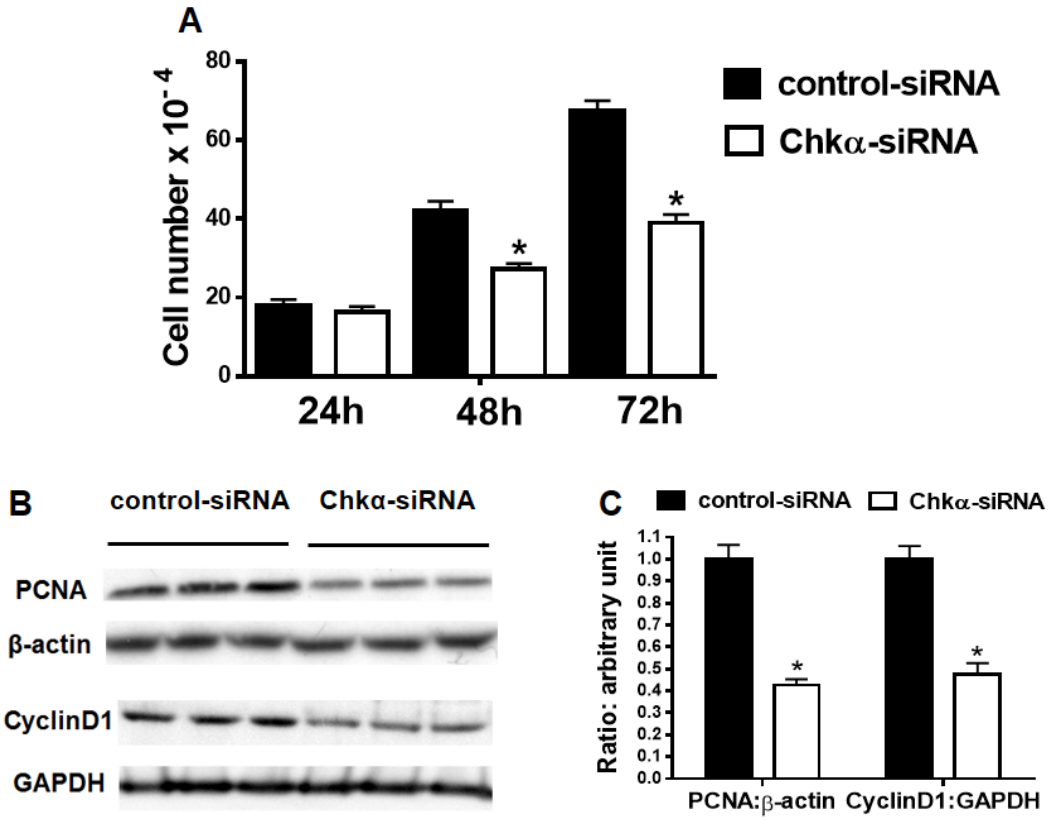

Figure 3.7 Decreased proliferation of MG-63 cells in which expression of the *Chkα* gene was silenced. *Chkα*-siRNA (100 nmol/L) was transfected into MG-63 cells. After 24, 48 or 72 h, the cells were harvested and viable cells that excluded Trypan blue were counted with a hemocytometer (A). Expression of PCNA and CyclinD1 were analyzed by immunoblotting of proteins from whole cell lysates 72 h after transfection (B). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as loading controls. The ratio of PCNA: β-actin and CyclinD1: GAPDH was calculated by densitometric analysis of the immunoblots using ImageJ 1.45s software (C). Data are means ± SEM from 3 independent experiments. * $P < 0.05$.

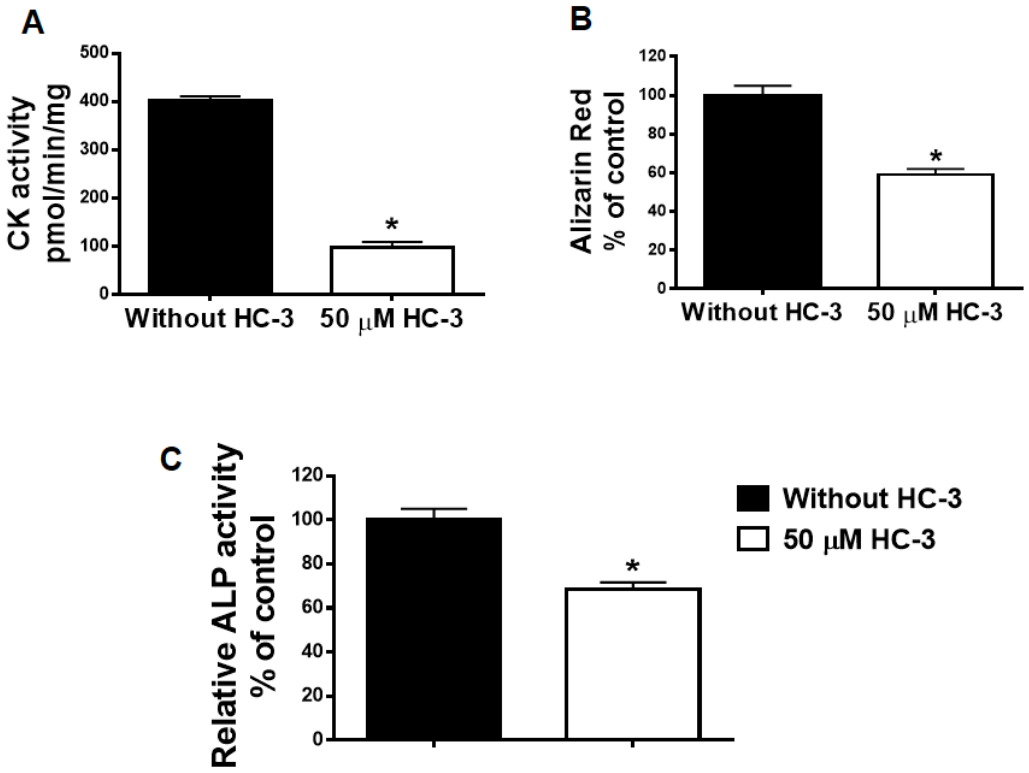

Figure 3.8 Inhibition of CK activity by HC-3 decreased mineralization capacity and alkaline phosphatase (ALP) activity in MG-63 cells. Confluent MG-63 cells were incubated in osteogenic growth medium supplemented with or without 50 μM HC-3 for 14 days. Cells were harvested and the activity of CK (A) and ALP (C) was measured in cell lysates. Cells grown in parallel wells were stained with 40 mM Alizarin Red. The bound stain was eluted with 10% cetylpyridinium chloride, and absorbance of supernatants was measured at 550 nm (B). Data in panels B and C are given as % of values from cells incubated without HC-3. Data are means ± SEM from 3 independent experiments. * $P < 0.05$.

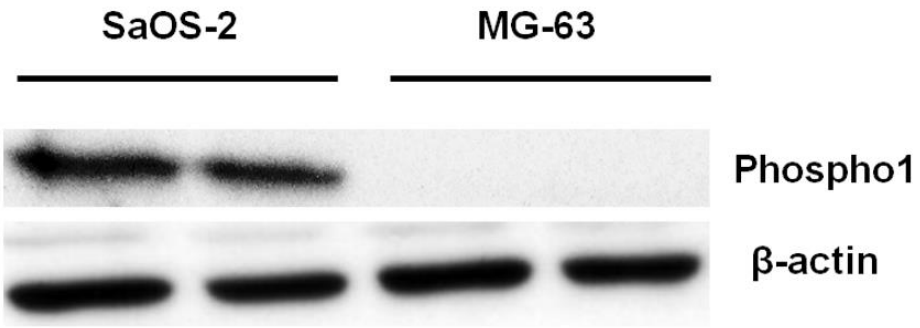

Figure 3.9 Phospho1 is not expressed in MG-63 osteoblast cells. 100 µg cytosolic proteins from SaOS-2 and MG-63 cell extracts were used to determine Phospho1 expression by immunoblot. β-actin was used as a loading control.

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Chapter 4

Summary and Future Directions

4.1 Conclusions

Chkb$^{-/-}$ mice were identified to carry a spontaneous homozygous genomic deletion within the *Chkb* gene and they develop postnatal hindlimb muscular dystrophy and neonatal forelimb bone deformation (1). Previous work in our laboratory has demonstrated that attenuated PC biosynthesis and mitochondrial dysfunction contribute to the muscular dystrophy (2). However, the reasons for the forelimb bone deformity were still unknown. In Chapter 2, we elucidated the mechanisms in which *Chkb* deficiency leads to the forelimb bone deformation.

Before investigating the potential mechanisms for the forelimb bone deformity, it was necessary to characterize the phenotype and determine the exact timing of the bone deformation. By skeletal staining and Micro-CT imaging, we observed that *Chkb*$^{-/-}$ mice have no gross skeletal abnormality, except for the radius and ulna specific deformity on the forelimb. The deformations are evident at birth, which raises the possibility that the deformities could take place prior to birth. Thus, we stained the embryonic forelimbs and observed both radius and ulna are deformed during late embryonic stage, by E17.5, and have reduced lengths starting ~E14.5. The deformation restricted to radius and ulna and occurring before birth leads to a speculation that the deformity is a consequence of abnormal limb patterning. *Hoxa11* and *Hoxd11* are two genes that control radius and ulna patterning during embryonic development, and the absence of these two genes completely blocks the formation of radius and ulna (3, 4). We did not observe a change in the expression of these two genes from both embryonic tissue and primary chondrocytes, suggesting that the bone deformation phenotype in *Chkb*$^{-/-}$ mice does not result from defective limb patterning. However, it would be beneficial to examine the expression localization and distribution of these two genes by utilizing the technique of *in situ* hybridization, as aberrant expression pattern of the Hox gene may also affect the skeletal development in embryonic stage (5).

The limb long bones are formed through endochondral bone ossification, a process in which bone does not result from direct mineralization, but rather is

formed through a cartilage intermediate template (6-9). Shorter and deformed radius and ulna of *Chkb*$^{-/-}$ mice are observed in the embryonic stage, implicating defective cartilage development. Thus, we investigated several key events in endochondral bone formation for radius and ulna. We first examined the physiology of growth plate, an essential cartilage structure that controls the lengthening of the long bones. We observed disorganized proliferation zones with loss of columnar organization and irregular chondrocyte morphology in radius and ulna of *Chkb*$^{-/-}$ mice, which implied defective chondrocyte differentiation and proliferation in their growth plates. Indeed, by performing immunohistochemistry of Sox9 and PCNA (proliferating cell nuclear antigen), we found chondrocytes in growth plates of the radius and ulna from *Chkb*$^{-/-}$ mice have impaired chondrocyte differentiation and reduced proliferation compared to wild-type littermates. Sox9 is a master transcription factor that regulates early chondrocyte differentiation, and heterozygous Sox9 mutant mice have abnormalities in primordial cartilage including bending of the radius and ulna (10). Thus, decreased Sox9 expression in radius and ulna of *Chkb*$^{-/-}$ mice could contribute to their bending phenotype. The reduction in chondrocyte proliferation correlates well with decreased bone lengths of the radius and ulna in *Chkb*$^{-/-}$ mice. During endochondral bone formation, chondrocyte cell proliferation is regulated by Indian hedgehog (Ihh) / parathyroid hormone-related protein (PTHrP) signalling pathway, and absence of either of these two genes in mice significantly inhibits the chondrocyte proliferation (11, 12). Thus, it would be interesting to examine the expression of these two molecules in the growth plate to determine if the reduced chondrocyte proliferation in radius and ulna of *Chkb*$^{-/-}$ mice results from dysregulation of Ihh/PTHrP pathway.

Another striking phenotype in the growth plate of the radius and ulna of *Chkb*$^{-/-}$ mice is the enlarged hypertrophic zone. In addition, radius and ulna in *Chkb*$^{-/-}$ mice also display delayed cartilage mineralization, which is manifested by delayed formation of the primary ossification center. This finding suggests that radius and ulna in *Chkb*$^{-/-}$ mice have defective replacement of cartilage by bone. Conversion of cartilage into bone requires efficient cartilage matrix degradation

and cartilage resorption, followed by establishment of the primary ossification center (6-9). Two matrix metalloproteinases (MMPs), MMP9 and MMP13, are mainly involved in degrading the cartilage matrix, and both *Mmp9*$^{-/-}$ and *Mmp13*$^{-/-}$ mice exhibit impeded cartilage matrix digestion that leads to expanded hypertrophic zones (13-15). Interestingly, we observed decreased gene expression of both *Mmp9* and *Mmp13*, as well as reduced MMP9 activity in radius and ulna of *Chkb*$^{-/-}$ mice, which is indicative of impaired cartilage degradation that leads to the enlarged hypertrophic zone phenotype in *Chkb*$^{-/-}$ mice. Osteoclasts are required for cartilage resorption, and mice that are deficient in osteoclast production have enlarged hypertrophic zones in their growth plates (16). We found decreased osteoclast recruitment to the cartilage / bone junction of radius and ulna in *Chkb*$^{-/-}$ mice by staining the osteoclast marker tartrate-resistant acid phosphatase (TRAP). The reduced osteoclast recruitment may be due to decreased MMP9 activity, as it has been shown that MMP9 activity is necessary for the migration of osteoclasts into hypertrophic cartilage during primary endochondral ossification (17). Fewer osteoclast numbers along the chondro-osseous junction in the radius and ulna of *Chkb*$^{-/-}$ mice could also be explained by impaired osteoclastogenesis. Therefore, it is necessary to determine if *Chkb* deficiency would attenuate osteoclast resorption activity in *Chkb*$^{-/-}$ mice. Furthermore, it would be interesting to generate osteoclast-specific *Chkb* knockout mice to investigate how the loss of *Chkb* would affect osteoclast function.

Choline deficiency was linked to abnormal bone development in turkeys and impaired bone remodeling in rats (18-20). Furthermore, two very recent human genetic studies showed that homozygous mutations in the gene coding for CTP: phosphocholine cytidylyltransferase (CT) are identified in patients with bone disease, suggesting that disruption of the CDP-choline pathway may affect skeletal development (21, 22). To determine how *Chkb* deficiency would affect the CDP-choline pathway in cartilage, we isolated primary chondrocytes from the combined radius and ulna and quantified the choline metabolites by mass spectrometry. We found the amount of choline was unchanged and only a small reduction (~10%) in cellular PC mass. It has been known that CK is not the rate-

limiting enzyme in the CDP-choline pathway to synthesize PC (23, 24). Furthermore, PC level was not changed in most tissues of *Chkb*$^{-/-}$ mice despite huge reduction in CK activity (1). Therefore, it is not surprising to observe a small reduction in PC mass in the chondrocytes from *Chkb*$^{-/-}$ mice. However, PCho level is dramatically decreased (~75%), consistent with the substantial reduction of CK activity in chondrocytes of *Chkb*$^{-/-}$ mice.

Generation of PCho has been shown as an essential process during DNA synthesis, and PCho has been used as a biomarker for proliferating cancer cells (25-27). Therefore, the reduced PCho in cartilage could explain the shorter radius and ulna in both embryonic and postnatal stage. In addition, PCho has been implicated in the function of neutral sphingomyelinase 2 (nSMase2), which hydrolyzes sphingomyelin to yield PCho and ceramide. Loss of nSMase2 in mice results in dwarfism, chondrodysplasia and limb bone deformation, which are similar to those exhibited in *Chkb*$^{-/-}$ mice, suggesting that PCho may play a role in the bone phenotypes in nSMase2-deficient mice (28-31). Therefore, considering the role of PCho in cell proliferation and its potential involvement in skeletal development, we speculate that reduced PCho in chondrocytes is still the most likely underlying cause for the developmental defects in the radius and ulna of *Chkb*$^{-/-}$ mice. However, more work is required to provide support to this proposed mechanism. For example, pregnant female mice could be fed PCho-enriched diet or injected with PCho to see if these treatments would reverse or at least improve the phenotypes of bone deformity and defective endochondral bone development. In addition, although *Chkα*$^{-/-}$ mice have early embryonic lethality (32), it would be interesting to knockout *Chkα* specifically in chondrocytes to see if it could also generate less PCho in cartilage and produce similar phenotypes as in *Chkb*$^{-/-}$ mice.

One mystery still puzzling us is that why the bone deformity only occurs in radius and ulna. We have demonstrated that both radius and ulna have various endochondral bone formation defects, which would significantly contribute to the deformation phenotype. However, it would be worthwhile to fully examine the endochondral bone development for each of the long bones in both limbs. It is

possible that other limb long bones only have very minor defects in endochondral formation, which is not severe enough to induce the deformity. Previous work in our laboratory has shown that *Chkb* is the predominant isoform in the hindlimb muscle, which partially explains why the hindlimb muscular dystrophy is more severe as a result of *Chkb* loss (33). However, we found *Chkb* is the major isoform in chondrocytes isolated from all the limb long bones. Thus, it appears to eliminate the possibility that the specific bone defects seen in radius and ulna are due to differential expression of the CK isoforms. Possibly, the unique anatomic features of murine radius and ulna, that is, being located in the distal forelimb as well as attached together to support the body weight, confer the fragility of deformity when *Chkb* is absent. Moreover, in our study, *Chkb* is globally deficient, which raises the concern that loss of *Chkb* in other tissues, such as muscle, tendon or even bone marrow fat could also contribute to the bone deformation phenotype. To eliminate such complications, it would be useful to generate cartilage-specific *Chkb* knockout mice to further investigate the role of *Chkb* in endochondral bone formation.

There is limited knowledge about PC metabolism in osteoblasts as well as the relationship between CK and the function of osteoblasts. In Chapter 3, we utilized two human osteoblast models, primary human osteoblast cells (HOB) and human osteosarcoma MG-63 cells, to characterize PC metabolism and explore the connections between CK and human osteoblasts function.

By metabolic radiolabeling, we first determined that the CDP-choline pathway is the only functional route for PC biosynthesis in human osteoblasts, and the PC production via PE methylation by PEMT was barely detectable. This finding agrees well with a previous study, which showed that PEMT pathway was not found in mouse calvarial tissues (34). Indeed, it has been shown that the PEMT pathway is only quantitatively significant for PC biosynthesis in liver (35).

Liver can act as a recipient of PC from plasma lipoproteins (36). In addition, recent studies showed that bone is also a quantitatively important organ

for the uptake of circulating lipoproteins (37-39). Consistent with the literature, we showed that, by *in vitro* uptake assay, lipoproteins can deliver PC to human osteoblasts in both HOB and MG-63 cells. It would be interesting to perform an *in vivo* assay, in which mice are injected with radiolabeled lipoproteins to assess the uptake of lipoproteins to bone tissues.

We next characterized CK in both HOB and MG-63 cells. Interestingly, we observed increased CK activity, CKα expression, and elevated level of PCho in MG-63 cells compared to HOB cells. In addition, we found that CKα is the predominant isoform in MG-63 cells but not in HOB cells. Increased CKα expression and CK activity, as well as higher PCho levels, have been detected in numerous human tumors and cancer cells (25-27). Thus, it is not surprising to observe higher CK activity, CKα expression and PCho level in osteosarcoma MG-63 cells than in HOB cells. Since CKα is the major contributor of total CK activity in MG-63 cells, we then utilized siRNA techniques to silence *Chkα* gene and determined its effect on CDP-choline pathway by quantifying the choline metabolites using mass spectrometry. Similar to what we found in the chondrocytes isolated from *Chkb*$^{-/-}$ mice, PC mass was unchanged despite a huge reduction of CK activity after siRNA treatment. This finding again supports the traditional picture that CK is not the rate-limiting enzyme in the pathway to produce PC. Choline accumulated and PCho was significantly reduced (~80%) after siRNA treatment. This finding is expected as choline could not be metabolized efficiently and thus leads to insufficient production of PCho after *Chkα* gene silencing.

Both CKα and PCho have been implicated in cell transformation and proliferation in carcinogenesis (25-27). Thus, we knocked down CKα in MG-63 cells to examine its effect on cell growth. We found that the cell proliferation was gradually attenuated after *Chkα*-siRNA treatment, along with the reduction in the expression of CyclinD1, a key protein involved in cell cycle progression (40). This finding suggests that the observed decreased cell proliferation is due to impaired cell cycle progression. Our data also correlate well with a previous

microarray study, which showed that over-expression of CKα in human HEK293T cells up-regulated CyclinD1 expression, whereas inhibition of CKα down-regulated CyclinD1 expression (41). The effect of decreased PCho in cartilage on bone growth in *Chkb*$^{-/-}$ mice is discussed above. Similarly, the attenuated cell growth in MG-63 cells could also result from reduced PCho due to *Chkα* silencing. To further support this mechanism, it would be interesting to supplement MG-63 cells with PCho during siRNA treatment to see if the PCho addition could reverse or improve the impaired cell proliferation.

Hemicholinium-3 (HC-3) has structural similarity to choline and is an effective inhibitor of CK (25). We have demonstrated that inhibition of CK by HC-3 significantly decreased the mineralization activity in MG-63 cells. CK can produce both PCho and phosphoethanolamine (42, 43), which are specific substrates of Phospho1, a recently identified phosphatase that is expressed in mineralizing tissue to generate inorganic phosphate (Pi) (44). Thus, we speculated that CK inhibition could deplete the substrate availability of Phospho1, so that insufficient Pi is generated for mineralization. However, we found Phospho1 is not expressed in MG-63 cells. Thus, it would be useful to utilize Phospho1-expressing cell system, such as SaOS-2 cells, to further investigate if Phospho1 plays a role in the decreased mineralization capacity induced by CK inhibition. Interestingly, we observed noticeable reduction of alkaline phosphatase (ALP) activity in MG-63 cells, the major phosphatase responsible for skeletal mineralization (44). However, we have not obtained data that could reveal the connection between CK and ALP. Future work should elucidate the mechanism of decreased ALP activity induced by reduced CK activity in MG-63 cells.

In Chapter 3, we chose to knock down CKα rather than CKβ, based on the finding that CKα is the major isoform in MG-63 cells. Suppose CKβ was knocked down in MG-63 cells, significant amounts of CK activity and PCho could still remain so that cell growth and mineralization would not be attenuated. In addition, HOB cells may be a more suitable cell system for the gene silencing study since they fully resemble the behaviour of primary osteoblast cells (45). As CKα and

CKβ contribute equally to the total CK activity in HOB cells, it would be worthwhile to knock down either CKα or CKβ in HOB cells to examine the effects on cell proliferation and mineralization. Furthermore, it would be interesting to generate osteoblast-specific CKα- or CKβ-deficient mice to provide additional insights into the role of CK in osteoblast functions. Nevertheless, the gene silencing study in MG-63 cells reveals the importance of CK for the growth and mineralization of human osteosarcoma cells and provides the insight that CKα could be a drug target to inhibit the bone tumor growth and treat bone cancer.

Previous work has shown that CKβ is required for normal muscle development (46). In Chapter 2, we demonstrated the essential role of CKβ for cartilage development during endochondral bone ossification. In Chapter 3, we revealed the importance of CKα for osteoblast function. Furthermore, I also observed that *Chkb*$^{-/-}$ mice, fed a high-fat diet, have reduced adipose tissue mass with smaller adipocytes. Interestingly, myoblasts in muscle, chondrocytes in cartilage, osteoblasts in bone and adipocytes in adipose tissue are all derived from mesenchymal stem cells (47). These findings could be a coincidence but it is likely that CK is involved in the mesenchymal stem cell differentiation so that loss-of-function of CK could affect all the differentiated tissues. Thus, it would be interesting to investigate the possible role of CK in mesenchymal stem cell differentiation, and this will provide some novel insights into the mechanisms in which CK regulates the development of muscle, cartilage, bone and adipose tissue.

In summary, this thesis describes the physiological importance of CKβ in murine endochondral bone formation and CKα in human osteoblasts function. We showed that the absence of CKβ in mice leads to various defects of endochondral bone formation in radius and ulna, which significantly contribute to their deformity. We also proposed that deceased PCho in cartilage is the potential underling cause for the defective radius and ulna development in *Chkb*$^{-/-}$ mice; we demonstrated that increased CK activity and CKα expression are characteristics of human osteosarcoma cells and CKα is required to maintain normal cell

proliferation and mineralization of osteoblast cells. We proposed that CKα is a candidate drug target for bone cancer treatment.

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