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The Role of Glycogen Synthase Kinase 3 Beta in Skeletal Development

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Graduate Program in Physiology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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THE ROLE OF GLYCOGEN SYNTHASE KINASE 3 BETA IN SKELETAL DEVELOPMENT

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(Thesis format: Integrated Article)

by

John Ryan James Gillespie

Graduate Program in Physiology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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The thesis by

John Ryan James Gillespie

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The role of glycogen synthase kinase 3 beta in skeletal development

is accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Abstract

Glycogen Synthase Kinase 3 beta (GSK-3β) is an important node of regulation in many cellular signaling pathways including insulin and Wnt. Previous work has shown that ubiquitous deletion of GSK-3β is lethal and causes various skeletal abnormalities, but it is not known whether these defects are skeleton-intrinsic. A second GSK-3 protein which shares almost complete homology, GSK-3α, has been shown to have completely overlapping roles with the β form in some cases and independent functions in other cases. Therefore examination of the role of GSK-3β in skeletal development is very poignant.

To examine the role of GSK-3β in skeletal development we used three models: 1. *Ex vivo* tibia organ cultures treated with pharmacological inhibitors, 2. *In vivo* cartilage-specific GSK-3β deletion, 3. *In vivo* bone-specific GSK-3β deletion. The tibia organ culture experiment revealed that inhibiting both GSK-3 forms caused increased bone growth. We also demonstrated that GSK-3 effects on chondrocyte proliferation were PI3K-dependent and regulated by GSK-3α whereas effects on hypertrophy were PI3K-independent and GSK-3β regulated. However when GSK-3β was deleted specifically in cartilage, there was no change in growth or growth plate morphology. One unique finding was that GSK-3α was upregulated in the pre-hypertrophic/hypertrophic region of mutant mice, presumably through a compensating mechanism.

Since the global knockout (KO) showed a skeletal phenotype and our cartilage-specific KO did not, we postulated that GSK-3β function in osteoblast was important for skeletal development. To test this we generated osteoblast-specific GSK-3β KO mice. These mice displayed skeletal phenotypes seen in the global deletion study plus several more since we were able to study postnatal development. These mice also displayed a metabolic phenotype. The mice had lower blood glucose and insulin but their pancreases were unchanged. Most interestingly, almost 100% of the mutant males died with high blood glucose and damage to their urogenital track, all reminiscent of type II diabetes development.
In conclusion we showed that cartilage GSK-3β is not important for skeletal development but both GSK-3s are required to regulate growth. We showed that osteoblast GSK-3β is important for skeletal development and whole body metabolic regulation.

Keywords

Glycogen synthase kinase 3 alpha, GSK-3β, diabetes, insulin, glucose, growth plate, osteoblast, chondrocytes, skeletal development
Co-Authorship Statement

Chapter 2: is adapted from Gillespie JR, Ulici V, Dupuis H, Higgs A, DiMattia A, Patel S, Woodgett JR, Beier F. Deletion of Glycogen synthase kinase-3beta in cartilage results in upregulation of Glycogen synthase kinase-3alpha protein expression. Endocrinology. 2011 May;152(5):1755-66. Epub 2011 Feb 15. J.R.G. performed most experiments and contributed to the study design and the writing of the manuscript. V.U., H.D, A.H., and A.D performed selected experiments. S.P and J.R.W created the GSK-3β floxed mice. F.B. contributed to the study design and the writing of the manuscript. All authors read and approved the submitted version of the manuscript.

Chapter 3: is adapted from Gillespie JR, Beaucage KL, Dupuis H, Wong SY, Pollmann SI, Holdsworth DW, Kream B, DiMattia G, Patel S, Woodgett JR, Beier F. Skeletal-specific deletion of Glycogen Synthase Kinase 3 beta (GSK-3β) delays postnatal growth and skeletal development. Resubmitted to JBMR December 2011. J.R.G. performed most experiments and contributed to the study design and writing of the manuscript. K.L.B. performed the μCT scans and performed the analysis with and assistance of S.I.P. and D.W.H. H.D and S.Y.W. performed selected experiments. B.K. created the Col I cre mice. G.D. assisted study design and writing of manuscript. S.P and J.R.W. created the GSK-3β floxed mice. F.B. contributed to the study design and the writing of the manuscript. All authors read and approved the submitted version of the manuscript.

Chapter 4: is adapted from Gillespie JR, Bush JR, Bell GI, Aubrey LA, Ferron M, Kream B, DiMattia G, Patel S, Woodgett JR, Karsenty G, Hess DA, Beier F. Skeleton-specific deletion of GSK-3β causes insulin sensitivity and male specific mortality due to possible development of type II diabetes. Submitted to JBMR December 2011. J.R.G. performed most experiments and contributed to the study design and writing of the manuscript. J.R.B. and L.A.A. performed selected experiments. G.I.B. performed the metabolic analyses and D.A.H contributed to the metabolic study design. M.F. performed the OCN carboxylation assay and
with G.K. advised on experimental design. B.K. created the Col I cre mice. G.D. assisted study design and writing of manuscript. S.P and J.R.W. created the GSK-3β floxed mice. F.B. contributed to the study design and the writing of the manuscript. All authors read and approved the submitted version of the manuscript.
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## Table of Contents

**CERTIFICATE OF EXAMINATION** .................................................................................................................. ii

Abstract .................................................................................................................................................................. iii

Keywords ............................................................................................................................................................... iv

Co-Authorship Statement ..................................................................................................................................... v

Acknowledgments ................................................................................................................................................ vii

Table of Contents ................................................................................................................................................ vii

List of Figures ..................................................................................................................................................... xiii

List of Appendices ............................................................................................................................................... xvi

List of Abbreviations and Symbols ..................................................................................................................... xvii

Chapter 1 .............................................................................................................................................................. 1

  1 Introduction .................................................................................................................................................... 1

    1.1 Skeletogenesis .......................................................................................................................................... 1

        1.1.1 Intramembranous Ossification .......................................................................................................... 1

        1.1.2 Endochondral Ossification ............................................................................................................... 2

        1.1.3 Cartilage Growth Plate ..................................................................................................................... 5

    1.2 Growth Plate Regulation ............................................................................................................................. 8

        1.2.1 Systemic Factors .............................................................................................................................. 8

        1.2.2 Local Factors ..................................................................................................................................... 10

        1.2.3 Transcription Factors ...................................................................................................................... 12

        1.2.4 Glycogen Synthase Kinase 3 Beta ..................................................................................................... 14

    1.3 GSK-3β ....................................................................................................................................................... 15

        1.3.1 Role of GSK-3β in bone development .............................................................................................. 20

    1.4 Bone as an endocrine organ ....................................................................................................................... 22

    1.5 Overall objectives and hypothesis ........................................................................................................... 25
1.5.1 Rationale ................................................................. 25
1.5.2 Overall Hypothesis .................................................. 26
1.5.3 Aims .............................................................................. 26
1.6 References ........................................................................ 26

Chapter 2 .................................................................................. 44

2 Deletion of glycogen synthase kinase-3beta in cartilage results in upregulation of glycogen synthase kinase-3alpha protein expression ........................................... 44

2.1 Introduction ....................................................................... 45

2.2 Material and Methods ....................................................... 47

2.2.1 Materials ...................................................................... 47
2.2.2 Mouse breeding and genotyping .................................... 47
2.2.3 Organ culture ............................................................... 48
2.2.4 Tibia staining ................................................................. 48
2.2.5 Histology and immunohistochemistry (IHC) .................... 48
2.2.6 Western blot analyses .................................................. 49
2.2.7 Statistical analysis ....................................................... 50

2.3 Results ............................................................................... 50

2.3.1 GSK-3 expression in the growth plate ......................... 50
2.3.2 GSK-3 inhibition increases bone growth and affects growth plate morphology ........................................ 50
2.3.3 GSK-3 inhibition rescues most effects of PI3K inhibition in organ culture ................................................. 53
2.3.4 Chondrocyte-specific ablation of GSK-3β in vivo ............ 56
2.3.5 GSK-3 inhibition increases chondrocyte proliferation ....... 59
2.3.6 Prehypertrophic cell cycle exit increased upon GSK-3 inhibition ................................................................. 64
2.3.7 β-catenin expression in prehypertrophic chondrocytes is upregulated upon inhibition of GSK-3 ......................................................... 64
2.3.8 Upregulation of GSK-3α protein in response to GSK-3β deletion in vivo69
3 Skeletal-specific deletion of Glycogen Synthase Kinase 3 beta (GSK-3β) delays postnatal growth and skeletal development

3.1 Introduction

3.2 Materials and methods

3.2.1 Materials

3.2.2 Mouse breeding and genotyping

3.2.3 Skeletal staining

3.2.4 Histology and immunohistochemistry (IHC)

3.2.5 Western blot analyses

3.2.6 Serum analysis

3.2.7 Micro-CT

3.2.8 Statistical analysis

3.3 Results

3.3.1 Col1a1-driven Gsk3b inactivation

3.3.2 Decreased weight, delayed postnatal bone growth, and altered growth plate morphology in mutant mice

3.3.3 Delayed skeletal development and ossification in KO mice

3.3.4 Suture closure and strength as well as snout length are altered in KO mice

3.3.5 Transiently increased bone formation in KO mice

3.3.6 GSK-3β regulates proliferation, cell cycle and apoptosis in skeletal cells

3.3.7 Increased expression of β-catenin in mutant skeletons

3.4 Discussion

3.5 References
4 Skeleton-specific deletion of GSK-3β causes insulin sensitivity and male specific mortality due to possible development of type II diabetes ........................................ 132

4.1 Introduction ........................................................................................................................................ 133

4.2 Material and methods ............................................................................................................................ 134

4.2.1 Materials ........................................................................................................................................ 134

4.2.2 Mouse breeding and genotyping ....................................................................................................... 135

4.2.3 Histology and immunohistochemistry (IHC) .................................................................................... 135

4.2.4 Western blot analyses ....................................................................................................................... 136

4.2.5 Blood glucose and serum measurements (Millipore Multiplex), glucose tolerance test and insulin tolerance test ........................................................................................................ 137

4.2.6 Statistical analysis ........................................................................................................................... 137

4.3 Results .................................................................................................................................................. 138

4.3.1 Metabolic changes and male specific mortality in skeleton-specific GSK-3β KO mice .................. 138

4.3.2 Serum insulin and leptin levels in skeleton-specific GSK-3β KO mice ...................................... 141

4.3.3 CollI-GSK-3β KO mice display increased insulin sensitivity .................................................... 149

4.3.4 Large bladder at time of death in male CollI-GSK-3β KO mice ............................................. 150

4.3.5 Damage to the corpus cavernosum in male CollI-GSK-3β KO mice ..................................... 156

4.3.6 Kidney damage and hydronephrosis in male CollI-GSK-3β KO mice ................................ 163

4.4 Discussion ........................................................................................................................................... 168

4.5 References .......................................................................................................................................... 171

Chapter 5 ................................................................................................................................................ 176

5 General Discussion and Conclusion ....................................................................................................... 176

5.1 Overall Rationale ............................................................................................................................... 177

5.2 Study 1 ................................................................................................................................................ 178

5.2.1 Experimental Rationale ................................................................................................................. 178
List of Figures

Figure 1.1 (1) Early endochondral bone formation ................................................................. 3
Figure 1.2 (2) Late Endochondral Bone Formation ................................................................. 4
Figure 1.3 (3) Mature bone ........................................................................................................ 6
Figure 1.4 (4) Growth plate zones ............................................................................................ 7
Figure 1.5 (5) Regulation of the growth plate ........................................................................... 13
Figure 1.6 (6) Structure of the two glycogen synthase kinase 3-beta proteins ....................... 16
Figure 1.7 (7) Insulin signaling pathway ..................................................................................... 18
Figure 1.8 (8) Canonical Wnt signaling pathway ....................................................................... 19
Figure 2.1 (9) Expression of GSK-3 proteins in the growth plate .............................................. 52
Figure 2.2 (10) Inhibition of GSK-3 increases bone growth and changes growth plate organization .................................................................................................................. 55
Figure 2.3 (11) Interaction of PI3K and GSK-3 signaling in organ culture ............................... 58
Figure 2.4 (12) Cartilage-specific deletion of GSK-3β ............................................................... 61
Figure 2.5 (13) GSK-3 inhibition increases proliferation in organ culture while GSK-3β deletion in cartilage has no effect on proliferation in vivo ................................................................. 63
Figure 2.6 (14) GSK-3 regulates p57 expression in the prehypertrophic zone in vitro while cartilage-specific GSK-3β deletion does not affect p57 expression in vivo ................................................. 66
Figure 2.7 (15) GSK-3β regulates β-catenin expression in the prehypertrophic zone .............. 68
Figure 2.7 (16) In vivo deletion of GSK-3β causes upregulation of GSK-3α protein in chondrocytes ......................................................................................................................................... 71
Figure 3.1 (17) Effective recombination ..................................................................................... 90
Figure 3.2 (18) – Bone-specific inactivation of the Gsk3b gene ................................................. 93
Figure 3.3 (19) – Reduced weight and growth plate changes on KO mice ......................... 97
Figure 3.4 (20) – Serum IGF and GH .................................................................................. 98
Figure 3.5 (21) – Delayed skeletal development and ossification on KO mice ............. 101
Figure 3.6 (22) – P0 delayed skeletal development ................................................................. 102
Figure 3.7 (23) – Delayed suture closure in KO mice ............................................................ 105
Figure 3.8 (24) – Increased bone formation in KO mice ...................................................... 108
Figure 3.9 (25) – Bone quality characterization ...................................................................... 109
Figure 3.10 (26) – ColII-GSK-3β KO trabecular bone analysis ........................................... 111
Figure 3.11 (27) – Increased osteoclast number in KO mice ............................................... 114
Figure 3.12 (28) – RANKL and OPG serum and tissue expression ....................................... 115
Figure 3.13 (29) – Proliferation, cell cycle and apoptosis ....................................................... 118
Figure 3.14 (30) – Expression of key transcription factors ..................................................... 121
Figure 4.1 (31) Metabolic phenotype (Glucose) in Coll-GSK-3β KO mice ....................... 140
Figure 4.2 (32) Individual glucose readings ............................................................................ 142
Figure 4.3 (33) Serum insulin and leptin are decreased in KO mice ................................. 144
Figure 4.4 (34) Individual insulin readings ............................................................................ 145
Figure 4.5 (35) GSK-3β ELISA ............................................................................................ 147
Figure 4.6 (36) Food intake .................................................................................................... 148
Figure 4.7 (37) Coll-GSK-3β KO mice show increased insulin sensitivity ...................... 152
Figure 4.8 (38) Pancreas is unchanged in mutant mice ........................................ 153

Figure 4.9 (39) Enlarged bladders in male mutant mice that expire prematurely .......... 155

Figure 4.10 (40) Male mutant mice display damage to the Corpus Cavernosum .......... 158

Figure 4.11 (41) Examples of damage ...................................................................... 160

Figure 4.12 (42) GSK-3β Immunohistochemistry of kidney and urogenital tract. ....... 162

Figure 4.13 (43) Evidence of kidney damage and hydronephosis in male mutants ....... 165

Figure 4.14 (44) Renal tubule damage ...................................................................... 167

Figure 5.1 (45) Signaling pathways within GSK-3β KO osteoblasts ......................... 188

Figure 5.2 (46) Effects on whole body metabolic signaling ...................................... 195
List of Appendices

Appendix A: Copyright permission ................................................................. 204

Appendix B: Animal use protocol ................................................................. 206
List of Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic proteins</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>BV</td>
<td>bone volume</td>
</tr>
<tr>
<td>C</td>
<td>celsius</td>
</tr>
<tr>
<td>C</td>
<td>control</td>
</tr>
<tr>
<td>cGKII</td>
<td>cGMP-dependent kinase II</td>
</tr>
<tr>
<td>CK</td>
<td>casein kinase</td>
</tr>
<tr>
<td>Col</td>
<td>collagen</td>
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<tr>
<td>Cont</td>
<td>control</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSH</td>
<td>dishevelled</td>
</tr>
<tr>
<td>E or e</td>
<td>embryonic</td>
</tr>
<tr>
<td>ECL</td>
<td>electrochemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factors</td>
</tr>
<tr>
<td>Fl</td>
<td>floxed</td>
</tr>
<tr>
<td>FoxO</td>
<td>forkhead box protein</td>
</tr>
<tr>
<td>FZD</td>
<td>frizzled</td>
</tr>
<tr>
<td>GC</td>
<td>glucocorticoids</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GMP</td>
<td>guanosine monophosphate</td>
</tr>
<tr>
<td>GSK</td>
<td>glycogen synthase Kinase</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
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<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>Hrp</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>ICAT</td>
<td>beta-catenin interacting protein</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin like growth factor</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>Ihh</td>
<td>indian hedgehog</td>
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<tr>
<td>IR</td>
<td>insulin receptor</td>
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<tr>
<td>K</td>
<td>knockout</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>KOH</td>
<td>potassium hydroxide</td>
</tr>
<tr>
<td>Lef</td>
<td>lymphoid enhancer-binding factor</td>
</tr>
<tr>
<td>Li</td>
<td>lithium</td>
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<tr>
<td>Lrp</td>
<td>lipoprotein receptor-related protiens</td>
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<tr>
<td>Mg</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>Ob</td>
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<tr>
<td>OC</td>
<td>organ culture</td>
</tr>
<tr>
<td>OCN</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>P</td>
<td>postnatal</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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PCNA  proliferating cell nuclear antigen
PDK  phosphoinositide dependent protein kinase
PI3K  phosphatidylinositol 3 kinase
PKB  protein Kinase B
PSA  Puck’s solution A
PTHRP  parathyroid hormone-related peptide
RIPA  radio immunoprecipitation assay
Runx2  runt-related transcription factor 2
S  serine
SE  standard error
SEM  standard error of mean
Ser  serine
SOX-9  sry (sex determining region y)-box 9
TBST  tris-buffered saline and tween 20
TCF  T cell-specific transcription factor
TRAP  tartrate-resistant acid phosphatase
TUNEL  terminal deoxynucleotidyl transferase
TV  trabecular volume
UDP  uridine diphosphate
VEGF  vascular endothelial growth factor
Wt  wildtype
Y  tyrosine
Chapter 1

1 Introduction

1.1 Skeletogenesis

Once thought to be just scaffolding for muscle attachment, the skeleton has been recently shown to have many other functions. One of these emerging roles is bone functioning as an endocrine organ to regulate energy metabolism, which will be discussed in great detail later. In retrospect, this role appears logical since the adult human skeleton makes up 14% of total body weight and therefore the development, growth and regulation of such a large portion of the body would require a large amount of energy.

The bones of our skeleton form through two different processes, endochondral and intramembranous ossification. The development and growth of the skeletal elements involves several cell types, e.g. osteoblasts (bone forming cells), osteoclasts (bone resorbing cells), and chondrocytes (cartilage forming cells). Coordination between cell types is very complex and involves both intrinsic and extrinsic factors.

1.1.1 Intramembranous Ossification

The mandible, clavicle, some of the facial bones and the flat bones of the cranium develop through intramembranous ossification (1). This process is triggered by epithelial-mesenchymal interactions, which cause the mesenchymal cells to differentiate into preosteoblasts. These cells condense, proliferate and differentiate into mature osteoblasts (1-3). The osteoblasts start to produce and deposit bone matrix, thus forming ossification centers. Intramembranous ossification does not need a cartilage template to form bone unlike endochondral ossification.
1.1.2 Endochondral Ossification

Endochondral ossification is the process by which most of the bones of the body are formed. This includes bones of the appendicular (long bones) and axial (vertebrae, ribs) skeleton (1,4,5). This process involves the production of a cartilage template that is replaced by bone.

Just like intramembranous ossification, endochondral bone formation is also initiated by the condensation of mesenchymal cells, which in this process differentiate into chondrocytes (4,6) (Figure 1.1). Chondrocytes are the cell type that produces cartilage matrix. This cartilage matrix is rich in type II collagen and the proteoglycan aggrecan (6). Surrounding these cartilage condensations is a layer of cells called the perichondrium (6)(Figure 1.1). The chondrocytes in the middle of the condensations exit the cell cycle and enlarge, becoming hypertrophic chondrocytes. These hypertrophic chondrocytes change their gene expression profile and start producing type X collagen. These cells also direct the cells in the surrounding perichondrium to differentiate into osteoblast and produce the bone of the bone collar (6) (Figure 1.2). Hypertrophic chondrocytes ultimately undergo apoptosis, leaving behind cartilage matrix scaffolding that is invaded by blood vessels (which are attracted by VEGF produced by hypertrophic chondrocytes). The blood vessels bring in pre-osteoblasts and osteoclasts that differentiate and convert the cartilage matrix to true bone, forming the primary ossification center (primary spongiosa) (6) (Figure 1.2). The ends of the bones are next invaded by blood vessel and ossified by osteoblasts, forming secondary ossification centres.
Figure 1.1 (1) Early endochondral bone formation
Figure 1.2 (2) Late Endochondral Bone Formation
The newly formed bone has a dense bone collar surrounding the outer surface of the middle (diaphysis) of the bone with spongy (trabecular) bone on the inside or marrow cavity. This primary ossification centre is capped at either end by a layer of cartilage called the growth plate, which, as the name suggests, is responsible for longitudinal growth. At the ends of the bone (epiphysis) is the secondary ossification centre, which is covered by another group of chondrocytes and cartilage called articular cartilage. Articular cartilage is primarily responsible for aiding articulation of joints by reducing friction and acting as a shock absorber (Figure 1.3).

1.1.3 Cartilage Growth Plate

The growth plate is essential in determining a person’s height. Situated at the either end of long bones they drive bone growth from the primary ossification centre in both directions. The growth plate has three distinct zones that can be identified by morphology, cell cycle progression, and gene expression patterns (Figure 1.4). The zone furthest from the centre of the bone is the resting zone where these chondrocytes serve as a reserve bank of chondrocytes for the rest of the growth plate (7). These cells can be identified morphologically as they appear small and round (Figure 1.4). In terms of cell cycle, these cells are not proliferating very rapidly. As the resting cells move closer to the diaphysis they start to proliferate very rapidly and produce cartilage matrix. Both these attributes force the chondrocytes to adopt their characteristic morphology, columns of flattened cells. These chondrocytes arrange in columns and form the proliferating zone (7) (Figure 1.4). The last zone of the growth plate, discussed previously, is the hypertrophic zone. This zone borders the primary ossification zone. This zone can be identified by the very large chondrocytes and changes in both cartilage
Figure 1.3 (3) Mature bone
Figure 1.4 (4) Growth plate zones
matrix produced and gene expression profiles (4,6,7) (Figure 1.4). There is also a transitional stage between the proliferating and hypertrophic called pre-hypertrophic that is unique and important. The chondrocytes of the pre-hypertrophic zone express genes important for cell cycle exit and a combination of genes expressed by proliferating and hypertrophic cells (8,9).

The rate at which the chondrocytes progress through these maturation stages determines the longitudinal bone growth. The enlargement of the hypertrophic cells, which increase 5-10 times in volume compared to proliferating chondrocytes, is responsible for a large portion of bone elongation (10,11). In fact, the chondrocyte height is responsible for up to 60% of longitudinal bone growth, while the remainder of growth is due to matrix synthesis and chondrocyte proliferation (10,12-15).

1.2 Growth Plate Regulation

Controlled and regulated growth is extremely important for an organism as too much or too little could be detrimental to survival. There are many regulatory molecules, from transcription factors, to secreted and to systemic factors, that affect various aspects of the growth plate chondrocyte’s life cycle and ultimately growth.

1.2.1 Systemic Factors

A variety of systemic factors affect bone growth some of which are growth hormone (GH) and insulin-like growth factor-1 (IGF-1), thyroid hormones (T3 and T4), and glucocorticoids (GC).
Growth hormone has long been known to effect growth, hence its name. Children with high GH levels exhibit gigantism whereas insufficient GH causes impaired growth as in children with mutation in the GH receptor (16). Most of the effects of GH on the skeleton are carried out by liver-derived IGF-1. In mice, a reduction in the amount of circulating IGF-1 results in a reduction of longitudinal growth (17), where administration of exogenous IGF-1 restores growth in mice and humans with GH receptor mutations (18,19). GH also has local action on the growth plate, stimulating growth, through to local production of IGF-1 (16). The main effect of GH on the growth plate chondrocytes is to stimulate proliferation (13) (Figure 1.5).

Thyroid hormones are systemic factors that affect skeletal growth. In humans with hypothyroidism, longitudinal bone growth is slowed, growth plates are thinned and chondrocyte hypertrophy is impaired (20). In contrast, hyperthyroidism causes increased growth velocity in children but also leads to premature growth plate fusion and short stature (21,22). Thyroid hormone has been shown to have several effects on chondrocytes including inducing maturation (23), and induction of hypertrophy without inducing proliferation (24-26). Mice heterozygous for an inactivating mutation in the thyroid hormone receptor (α) displayed longitudinal growth retardation and impaired chondrocyte hypertrophy (27). So it appears that the GH/IGF axis effects the proliferation of growth plate chondrocytes whereas the thyroid hormone affects hypertrophy (Figure 1.5).

Another example of a systemic factor is represented by glucocorticoids (GCs). Various childhood diseases require GC treatment which causes decreased bone volume and growth retardation (28). On the contrary, GC deficiency is associated with tall stature
GC is a potent inhibitor of chondrogenesis as treatment with dexamethasone inhibits chondrocyte proliferation and matrix production (30).

1.2.2 Local Factors

As well as being controlled by systemic signals, the three zones of the growth plate also regulate themselves through locally secreted factors. Some of the local secreted factors that control bone growth are Indian hedgehog (Ihh), parathyroid hormone-related peptide (PTHrP), bone morphogenic proteins (BMP), Wnt family proteins, and fibroblast growth factors (FGF).

Indian hedgehog (Ihh) is a secreted factor that is produced by prehypertrophic chondrocytes. Ihh stimulates chondrocyte proliferation and inhibits chondrocyte hypertrophy (31-34). The inhibition of hypertrophy is dependent on Ihh-stimulated upregulation of parathyroid hormone-related peptide (Figure 1.5). Another function of Ihh is to cause mesenchymal cells in the perichondrium to differentiate into osteoblasts and form the bone collar (33,35).

Parathyroid hormone-related peptide (PTHrP) is expressed in perichondral and early proliferating chondrocytes and diffuses away from the site of production to regulate cells expressing PTH/PTHrP receptor (36). The receptor is expressed in low amounts in proliferating chondrocytes and expressed in much greater amounts by cells that are initiating an inhibition of proliferation. The role of PTHrP is to maintain the chondrocytes proliferating and prevents hypertrophy (37) (Figure 1.5). As chondrocytes proliferate and move farther from the site of PTHrP production, they lose PTHrP
signaling, stop proliferating, undergo the early steps of hypertrophy, and start producing Ihh. The Ihh produced then feeds back to maintain the production of PTHrP.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily and play many roles in skeletogenesis. Proliferating chondrocytes express BMP7 whereas the pre-hypertrophic and hypertrophic chondrocytes express BMP6 (38). BMP signaling promotes chondrocyte proliferation in the growth plate (39,40) (Figure 1.5). The role that BMPs play in regulating the hypertrophic chondrocytes is much less clear, although it appears important for normal rates of onset and progression of hypertrophy (4). BMP signaling induces Ihh expression and the reverse is also true, although effects on the growth plate are independent of each other (39,41).

The Wnt family of secreted proteins and their signaling pathway play an important role in growth plate regulation. Several Wnt members are expressed in the growth plate with the highest expression in the proliferating and hypertrophic zones (42). This signaling within the growth plate is important for chondrocyte survival, proliferation and hypertrophy (43-45) (Figure 1.5). When the target of the canonical Wnt pathway, β-catenin, is constitutively active, it suppresses hypertrophy in immature chondrocytes but promotes terminal differentiation in mature chondrocytes (46). In the early proliferating zone, the canonical Wnt signaling effect on survival appears to be downstream of Ihh, however in late proliferating it appears to be independent of Ihh (44). Furthermore, the Wnt antagonist, secreted frizzled receptor 1, is expressed in prehypertrophic chondrocytes and likely acts as a regulator of Wnt’s effects on chondrocyte maturation (47).
The last of the local factors that will be discussed here are fibroblast growth factors (FGFs). The proliferating and early hypertrophic chondrocytes express fibroblast growth factor receptor-3 (FGFR3), where its activation leads to an inhibition of chondrocyte proliferation and acceleration of hypertrophy (48). Activating mutations in the human FGFR3 gene lead to short-limbed dwarfisms including the most common form of dwarfism, achondroplasia (49). Treatment of limb explant cultures with FGF2 leads to a reduction in the number of cells expressing Ihh (48). Forced expression of Ihh inhibits FGF2’s acceleration of hypertrophy, which suggests that FGF acts upstream of Ihh. However, the effects of FGF2 on proliferation are not counteracted by the forced expression of Ihh, which indicates that FGF suppression of proliferation is independent of Ihh. In fact, the effects of FGF signaling on chondrocyte proliferation, Ihh expression and hypertrophy are antagonized by BMP signaling (48)(Figure 1.5).

1.2.3 Transcription Factors

The transcription factor Sox9 is thought of as the major chondrocyte transcription factor. Sox9 is essential for converting cells of condensation into chondrocytes and acts at every stage of chondrocyte differentiation (6). Sox9 is expressed by proliferating but not hypertrophic chondrocytes and induces components of the cartilage matrix, including collagen type II and aggrecan (50). Study of Sox9 in vivo has been difficult as deletion in undifferentiated limb bud mesenchymal cells results in complete failure of chondrogenesis. When Sox9 is deleted in chondrocytes expressing collagen type II, the cells fail to undergo further differentiation to reach the stages of the growth plate (51). These studies and others show that Sox9 is required for normal rates of chondrocyte proliferation and delaying the onset of hypertrophy (4) (Figure 1.5). There is evidence
Figure 1.5 (5) Regulation of the growth plate
that Sox9 is the target downstream of PTH/PTHrP receptor to mediate the inhibition of hypertrophy in response to PTHrP (4). There is also a human condition called campomelic dysplasia that is caused by defects in Sox9. This condition is characterized by craniofacial defects, kyphoscoliosis, bowing and angulation of long bones among other features (52,53).

Originally the transcription factor Runx2 was thought of as the major osteoblast specific transcription factor as mice with deleted Runx2 have no osteoblasts (54,55). However, it is now clear that Runx2 is also important in growth plate chondrocyte regulation as it drives proliferative chondrocytes to differentiate further into hypertrophic chondrocytes (50) (Figure 1.4). Runx2 is the transcriptional activator of hypertrophic chondrocyte markers such as collagen type X and also activates the Ihh promoter (56). Runx2 expression is inhibited by PTHrP signaling, which is thought to contribute to the ability of PTHrP to inhibit hypertrophy (57). Furthermore, the activity of Runx2 is repressed by Sox9 through a direct interaction between the two transcription factors (58).

1.2.4 Glycogen Synthase Kinase 3 Beta

One key regulatory molecule that has been implicated in many if not all of the factors described above is glycogen synthase kinase 3 beta (GSK-3β). GSK-3β has been implicated in regulation of the Wnt/β-catenin pathway (59-62), the insulin/IGF pathway (63-66), thyroid hormone signaling (67), glucocorticoid signaling (68), Runx2 activity and stability (69,70), BMP signaling (71,72), Sox9 activity and expression (43,73), PTH signaling (74), Ihh signaling (44,75), and FGF signaling (76). GSK-3β is an important
node of regulation for many pathways important to skeletal development and therefore requires a closer examination.

### 1.3 GSK-3β

Glycogen Synthase Kinase 3 was first discovered in 1978 by Sir Philip Cohen from the University of Dundee, Scotland (77). Students of Sir Cohen then partially purified and characterized GSK and identified 3 serine residues on glycogen synthase (GS) that are targeted by GSK-3 (78,79). The final purification to homogeneity was performed by two more of his students, Brian Hemmings and James Woodgett (80,81). Dr. James Woodgett is now a leader in GSK-3 research and contributed to the following studies by providing the floxed GSK-3β mice.

GSK-3 is a highly conserved protein kinase for which genes have been identified in every eukaryotic genome studied to date (82). In mammals, GSK-3 is encoded by two genes, $Gsk3\alpha$ and $Gsk3\beta$. $Gsk3\alpha$ encodes a protein 51 kDa in size whereas the GSK-3β protein is 47 kDa (83) (Figure 1.6). The two GSK-3 proteins share almost complete sequence identity between their protein kinase domains and a high degree of conservation around their ATP binding sites (83). The greatest structural difference between the two is an amino-terminal, glycine rich region specific to GSK-3α that accounts for the 4 kDa size difference between the proteins. Both mammalian GSK-3 proteins are inhibited by amino-terminal serine phosphorylation, GSK-3α on S21 and GSK-3β on S9 (Figure 1.6).

There are at least two ways in which GSK-3β activity is inhibited physiologically, through direct phosphorylation (Ser9) by a variety of kinases (65) or through disruption of a protein complex involved in canonical Wnt signaling (84). Several protein kinases
Figure 1.6 (6) Structure of the two glycogen synthase kinase 3 proteins
are capable of inactivating GSK-3. The most highly studied is PKB/Akt (85,86), which inhibits GSK-3 in response to insulin (or IGFs) (65) (Figure 1.7). The secreted glycoproteins of the Wnt family initiate a canonical signaling cascade following binding to 2 membrane-bound receptors (FZD and Lrp5 or 6). Canonical Wnt signaling disrupts the protein complex of adenomatous polyposis coli (APC), Axin, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK-3) that in the absence of Wnt ligands phosphorylates β-catenin (Figure 1.8). Phosphorylation of β-catenin results in ubiquitination and degradation of β-catenin. Upon Wnt signaling, this process is inhibited (disruption of destruction complex), β-catenin accumulates and translocates to the nucleus where it binds to the transcription factors lymphoid enhancer-binding factor 1 (LEF) / T cell-specific transcription factor (TCF) and ultimately affects gene transcription (87)(Figure 1.8). GSK-3 is thus the common focal point of several integrated signaling systems and a wide selection of cellular stimulants.

GSK-3 is a serine/threonine kinase capable of phosphorylating a large number of substrates including glycogen synthase, from where its name originated, and β-catenin. It has been proposed that there may be two pools of GSK-3β; one involved in canonical Wnt signaling and another involved in other pathways and directly phosphorylated (59,88). It has also been proposed that GSK-3α and GSK-3β are functionally redundant and may be able to compensate for lack of the other (62), whereas others have shown differential roles for the two isoforms (89). These differences are likely due to tissue- or stimulus-specific effects. Tissue specificity of GSK-3 is a widely debated topic in the GSK-3 field recently and is a logical explanation for the numerous functions (differentiation, polarity, migration, apoptosis, development, metabolism, translation, and
Figure 1.7 (7) The Insulin signaling pathway
Figure 1.8 (8) The canonical Wnt signaling pathway
transcription) and various pathways regulated by GSK-3. While GSK-3 is a key regulator in many tissues throughout the body, including brain, heart, and bone, it is likely that it does not have the same cellular functions or is regulated by the same upstream pathways in all cells/tissues. Tissue specificity will depend on cellular receptors, state, regulatory molecules, transcription factors, and cellular machinery present.

As a result of the versatility of GSK-3, it has been studied extensively in many pathological conditions such as cancer (90,91), brain disorders such as schizophrenia and bipolarism (92,93) and diabetes (94,95). GSK-3β is aggressively being targeted as possible drug target to treat the aforementioned disorders (96).

1.3.1 Role of GSK-3β in bone development

One of the most studied bone regulatory pathways is canonical Wnt signaling through β-catenin, which is required for normal chondrocyte and osteoblast differentiation (43,97,98). Another pathway that has been identified as crucial for bone development is insulin/insulin like growth factor (IGF) (99-101). These pathways and others important in skeletal development are directly regulated by GSK-3.

One of the earliest and best-characterized GSK-3 inhibitors, which is still widely used, is lithium (Li) (102,103). The mode of inhibition by lithium is competition for Mg$^{2+}$ binding sites, which are required for GSK-3β activation (104). Treatment of wild-type mice with lithium significantly increased bone mass in vivo (105). In addition, several ATP competitive inhibitors have been developed, such as SB-216763 and SB-415286 (106,107). These and other inhibitors have been used in various in vitro and in vivo experiments to determine the molecular and physiological roles of GSK-3. New
inhibitors are being developed and tested on a continuing basis. An orally bioavailable GSK-3α/β dual inhibitor was used to show an increase in osteoblast differentiation in vitro and increase bone mass and strength in vivo in rats (108). The use of inhibitors identified GSK-3 as an important molecule for bone development and possibly as a target for osteoporosis treatment.

Many transgenic mouse models involving various components of the canonical Wnt pathway display skeletal phenotypes. Mice overexpressing Wnt4a in chondrocytes are dwarfed with craniofacial abnormalities and delayed ossification of lumbar vertebrae (109). Two mouse loss-of-function models for the Lrp5 gene displayed decreased bone mass (110,111), whereas gain-of-function mice conversely had high bone mass (112,113). β-catenin was examined in many mouse models. The total β-catenin knockout is embryonically lethal at E7.5 (114), and therefore other strategies were adopted to study β-catenin function in vivo. β-catenin deletion in mesenchymal precursors resulted in enhanced chondrogenesis (115,116). However, β-catenin deletion later in development under control of the Col2a1 promoter caused decreased chondrocyte proliferation and maturation (43). These models indicate a differential role for β-catenin at different stages of chondrocyte maturation. A group produced a mouse model that overexpressed ICAT, a β-catenin inhibitor, under the Col2a1 promoter (117). These mice were born with normal size, but became increasingly runted and showed decreased chondrocyte proliferation and increased hypertrophic chondrocyte apoptosis.

Finally, several groups have examined whether genetic manipulation of GSK-3β causes skeletal defects (118,119). The GSK-3β total KO was originally described to be embryonically lethal (118). In a later study, mutant animals survived to P0 with cleft
palates, bifid sternum and delayed ossification of the sternum, skull, ear bones, and cranial base (119). Heterozygous deletion of GSK-3β also caused a skeletal phenotype, namely increased ossification, clavicle abnormalities and increased bone reabsorption (69). The total and heterozygous KO of GSK-3β seemed to display opposite phenotypes, where heterozygous mutants showed increased ossification and null animals displayed delayed ossification. Importantly, these findings were obtained with conventional KO mice where Gsk3b was inactivated in all cells of the body; the role of this gene in individual skeletal cells are therefore not understood.

All these findings point towards an important role of GSK-3β in skeletal development; however the specific role in the various pathways controlling skeletal development is still unclear. The developed mouse models to study GSK-3β in skeletal development have shown an equally complicated pattern of phenotypes that seem contradictory to each other, increased bone formation versus delayed bone development and ossification.

1.4 Bone as an endocrine organ

Recently a new function has been emerging for bone, as an endocrine organ. For many years it has been known that certain hormones affect bone. However, hormone systems generally operate with a negative feedback loop, suggesting that bone would need to respond with a hormone of its own. To date, the only such hormone is osteocalcin (120). The search for the elusive bone hormone started by narrowing the search to proteins that are specific to osteoblasts and are secreted into circulation. This simplified matters greatly as osteocalcin is one of only a handful of proteins that is osteoblast-specific and furthermore is secreted into the circulation (121,122). The first osteocalcin knockout mice were produced in 1996 and shown to have high bone mass but no mention of any
metabolic changes was made at this time (123). It was not until 2007 that osteocalcin would be identified as a bone hormone affecting metabolism (120). Osteocalcin undergoes posttranslational modification where glutamic acid residues are carboxylated (Gla) (121). This carboxylation gives osteocalcin a high affinity for mineral ions and anchors it in bone mineral matrix. However, both gain- and loss-of-function studies have failed to determine a role for osteocalcin in extracellular matrix in vivo (123,124).

The publication that first identified osteocalcin as a bone hormone also identified a regulator of the metabolic effects, Esp (120). Esp encodes a protein that is expressed in osteoblasts and Sertoli cells called receptor-like protein tyrosine phosphatase (OST-PTP) (125). The Esp−/− mice where born with a 3-fold reduction in blood glucose when measured prior to first feeding, and 35% of the KO pups died before weaning (120).

Even though the Esp gene is only expressed in osteoblast and Sertoli cells, the Esp KO mice displayed changes in their pancreas and fat. The mice had increased pancreatic insulin, islet size, and beta cell mass as well increased serum insulin levels and insulin sensitivity (120). The KO mice also showed decreased gonadal fat pads and increased adiponectin levels (an adipokine enhancing insulin sensitivity) (120).

Osteocalcin (OCN)−/− mice were also examined for metabolic changes and were shown to have a completely opposite phenotype to the Esp KO. The OCN KO mice had increased blood glucose and fat mass (120). They also displayed decreased serum insulin, insulin sensitivity and adiponectin (120). To test whether OCN was the active hormone, a variety of co-culture assays were performed using osteoblast, β-cells, and adipocytes. The experiments showed that OCN was able to increase insulin secretion from the β-cells
as well as adiponectin from adipocytes. However, OCN expression was not altered in the Esp KO mice, suggesting that Esp must be affecting OCN activity. From this the researchers discovered that the active bone hormone was the uncarboxylated form of osteocalcin, which was regulated by ESP (120). This ability of uncarboxylated osteocalcin to regulate pancreas insulin secretion/blood glucose regulation and adipocyte adiponectin production was confirmed in wild-type mice (126).

The new role for osteocalcin as the bone hormone did not actually show a possible link to GSK-3β until 2010 when two papers were published by two different groups (127,128). Although the connection to GSK-3β was not explicitly established in these publications, they both characterized mice that had the insulin receptor deleted specifically in osteoblasts. Given the well-documented regulation of the insulin pathway by GSK-3β, the connection between the two can be made (63-66,129). Osteoblast-specific insulin receptor (Ob-IR) KO mice have increased blood glucose and decreased serum insulin (127). Insulin tolerance was decreased and likewise insulin resistance increased (127,128). It was also shown that the Ob-IR KO mice had increased fat and decreased bone acquisition (128). Both papers discovered different mechanisms through which the insulin receptor regulated the undercarboxylated osteocalcin. One paper showed that the insulin receptor inhibits FoxO1, which regulates bone resorption by osteoclasts (127). This resorption, and more specifically the acidity of the resorption fluid, decarboxylated the osteocalcin in the bone matrix and released it to circulation (127). This is an interesting finding as it ties the energy extensive process of bone remodeling to energy metabolism. The other group found that the insulin receptor inhibited the transcription
factor Twist2, which in turn inhibited Runx2. The level of Runx2 controlled the amount of undercarboxylated osteocalcin released (128).

1.5 Overall objectives and hypothesis

1.5.1 Rationale

By understanding the signaling pathways involved in bone development and maintenance one can start to understand and manipulate pathways involved in the development of disease states. It has been shown numerous times that GSK-3β regulates numerous pathways and molecules involved in skeletal development and growth by regulating various cellular activities such as proliferation, survival, cell cycle progression, and angiogenesis. It is interesting that typically GSK-3 negatively regulates pathways and acts like a “brake” on the anabolic processes. Therefore, removal or release of the brake through inhibition or genetic manipulation should accelerate proliferation, survival, cell cycle progression, and angiogenesis. GSK-3 inhibitors have demonstrated the importance of GSK-3β in bone mass regulation and identified this molecule as a therapeutic target for osteoporosis. Furthermore, genetic deletion of GSK-3β causes mortality and skeletal malformations. Finally, the heterozygous GSK-3β mice display a high bone mass phenotype. Highlighted here is the direct evidence of GSK-3β; however, there is a plethora of indirect evidence from studies manipulating pathways components either upstream or downstream of GSK-3β. These studies usually focus on components of the insulin/IGF or Wnt/β-catenin pathways of which I mostly focus on as well. However, two recent reviews compiled lists of extracellular factors shown to regulate GSK-3 activity as well as a list of the known GSK-3 substrates of which there is greater
than 100 (130,131). This multitude of pathways and substrates as well as the complexity of signaling regulation highlights the biological importance of GSK-3β.

No one to date has been able to study the role of GSK-3β in postnatal bone development as the total GSK-3β KOs are lethal and no one knows if the skeletal effects are skeletal cell autonomous. I therefore set out to produce cartilage- and bone-specific GSK-3β deletions to study the role of GSK-3β in skeletal development.

1.5.2 Overall Hypothesis

Deletion of GSK-3β in specific skeletal tissue, cartilage and bone, will cause an increase in cell proliferation and survival altering bone growth and development.

1.5.3 Aims

Aim 1: To determine the role that GSK-3β plays in growth plate function, endochondral bone formation and bone growth.

Aim 2: To determine the role of GSK-3β signaling in osteoblasts during postnatal bone development.

Aim 3: To investigate the whole body metabolic effects of deleting GSK-3β in osteoblasts.

1.6 References


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Chapter 2

2 Deletion of glycogen synthase kinase-3beta in cartilage results in upregulation of glycogen synthase kinase-3alpha protein expression

2.1 Introduction

Most bones develop through endochondral ossification, in which a cartilage scaffold is first produced by chondrocytes and then converted to calcified bone tissue by bone-forming cells, osteoblasts. Chondrocytes of the growth plate are responsible for longitudinal growth of endochondral bones (1,2). The growth plate is divided into three distinct zones that can be identified by either historical features, rate of cell cycle progression or marker gene expression. The “resting” zone is farthest from the mid bone (diaphysis) and consists of small chondrocytes with little cytoplasm and relatively low rates of proliferation. Some of these cells mature into “proliferative” zone chondrocytes that undergo rapid proliferation, resulting in flattened columnar cells surrounded by cartilage matrix. The proliferating chondrocytes then withdraw from the cell cycle and differentiate further into prehypertrophic and ultimately “hypertrophic” chondrocytes residing at the interface between growth plate cartilage and ossified bone. These hypertrophic cells represent the terminal differentiation stage and secrete a large amount of matrix and regulatory proteins. Finally, the hypertrophic cells die by apoptosis and leave behind a cartilage matrix that is invaded by blood vessels accompanied by osteoblast (bone-forming) and osteoclast (bone-resorbing) precursors, ultimately resulting in the replacement of cartilage by bone tissue (3-8).

The rate in which the chondrocytes progress through the zones of the growth plate determine the longitudinal growth of bone. This rate is consequently extremely important for normal skeletal development and final height in humans and is therefore tightly regulated both intrinsically and extrinsically by a complicated network of signaling pathways(2,9-11).

Many of these signaling pathways have been linked to the regulatory kinase glycogen synthase kinase three, GSK-3. Two pathways with relevance to this study are insulin or insulin-like growth factor (IGF)/PI3K/AKT/GSK-3 (12-16) and Wnt/GSK-3/β-catenin (14,17,18). GSK-3 is a serine/threonine kinase capable of phosphorylating a large number
of substrates including glycogen synthase, from where its name originated, but its role in physiology has expanded exponentially since its discovery. GSK-3 is an unusual kinase in that it is constitutively active and usually negatively regulates pathways (19-21). Mammals have two GSK-3 proteins, GSK-3α and GSK-3β (encoded by different, highly homologous genes), with masses of 51kDa and 47kDa, respectively. Regulation of GSK-α and β can occur through at least two mechanisms: a) through direct phosphorylation (Ser21 and Ser9 of GSK-3α and β, respectively), for example by the phosphatidylinositol 3-kinase (PI3K)/AKT (12) signaling pathway, or b) through disruption of the protein complex involved in canonical Wnt signaling (22). Potential crosstalk between these two pathways is highly debated as is the relationship between the two GSK-3 proteins, GSK-3α and β. Many studies have demonstrated that phosphorylation of GSK-3 does not affect β-catenin levels whereas other studies have suggested it can (23-27). Likewise there is evidence for both overlapping and distinct roles of GSK-3α and GSK-3β (16,17,28-31). Tissue-specific roles and relations could explain some of these seemingly contradictory results (16,32).

Several groups have examined how genetic manipulation of Gsk3b affects the skeleton (33,34). The germline homozygous deletion of Gsk3b shows a variable phenotype depending on the genetic background and can result in embryonic lethality (33) or survival to P0 with cleft palate, bifid sternum and delayed ossification of the sternum, skull, ear bones, and cranial base (34). Heterozygous deletion of Gsk3b also causes a skeletal phenotype with increased ossification, clavicle abnormalities and increased bone resorption (35). It would appear that these opposing skeletal phenotypes are Gsk3b dosage-dependent effects; however, all these are phenotypes based on germline loss of Gsk3β. Consequently it is unclear whether these skeletal phenotypes are cell or tissue autonomous. Interestingly, GSK-3α global KO mice have also been created; these are viable and fertile with similar body mass compared to controls (36) but display abnormalities in glucose metabolism and brain structure (16,36,37) without any described skeletal abnormalities. The two global KO models suggest that GSK-3β may play a more important role in skeletal development than GSK-3α. Here we address the role of GSK-3
signaling in chondrocytes using an organ culture system and cartilage-specific inactivation of the Gsk3b gene.

2.2 Material and Methods

2.2.1 Materials

The following antibodies were utilized in this study: actin A5441 (Sigma); Cyclin D1 RM-9104-S1 (Neomarkers); goat anti-rabbit hpr sc-2004, goat anti-mouse hpr sc-2005, p57/Kipp2 sc-8298 (Santa Cruz); GSK-3β #9315, pGSK-3β #9336, GSK-3α #9338, pGSK-3α & β #9331, β-catenin #9562 (Cell Signaling). General chemicals and supplies were purchased from Sigma and VWR, organ culture reagents were from Invitrogen.

2.2.2 Mouse breeding and genotyping

Mice homozygous for floxed Gsk3b alleles (Gsk3b$^{fl/fl}$) have been described previously (16, 38). Gsk3b$^{fl/fl}$ mice were crossed with mice expressing cre recombinase under control of the cartilage-specific mouse Col2a1 promoter, donated by Drs. R. St-Arnaud and G. Karsenty, that we previously utilized in our lab (39, 40). Mice heterozygous for the floxed Gsk3b allele and expressing Col2a1 cre were backcrossed with homozygous Gsk3b floxed mice. The offspring from these crosses were analyzed. Mice were exposed to a 12 hr light-dark cycle and fed tap water and regular chow ad libitum. All procedures involving animals were approved by the University of Western Ontario Animal Care and Use Committee. PCR genotyping was performed from ear notch DNA using primers 5’-GGGGCAACCTTAATTTCATT-3’ (forward) and 5’-TCTGGGCTATAGCTATCTAGTAACG-3’ (reverse) for GSK-3β for 30 cycles of 96 °C 55sec, 56.5°C 45 sec, 68 °C 2:45min to amplify. The cre transgene was detected using the primers 5’-CACACTGTGTAGTGCTTCGT-3’ (forward) and 5’-CCTCCAAACCATCCAAGAT-3’ (reverse) using 40 cycles of 95 °C 45sec, 58 °C 30sec, 72 °C 1min.
2.2.3 Organ culture

Tibiae were isolated from embryonic day 15.5, e15.5, mice and cultured for 6 days in serum-free medium containing alpha MEM, ascorbic acid, β-glycerophosphate, bovine serum albumin, glutamine, and penicillin-streptomycin as described, without exogenous growth factors (15, 41). Following dissection, tibiae were incubated in medium overnight, then treated with DMSO (control) or the GSK-3 inhibitor SB415286 (SB86; 10 µM), PI3K inhibitor LY 294002 (10 µM LY), or a combination of the two inhibitors. Please note that SB86 inhibits both GSK-3 proteins (42). Media and inhibitor were changed every 48 hrs. Length of tibiae was measured before start of treatment and at the end of 6 days. Each independent experiment consisted of 5-6 tibiae per treatment; data represent averages from at least 3 independent experiments. These bones were then either stained with Alcian Blue/Alizarin Red or prepared for paraffin embedding, sectioned and analyzed by immunohistochemistry (IHC).

2.2.4 Tibia staining

Tibia staining was performed as described previously (39, 43). Organ culture tibia isolated from e15.5 embryos were dehydrated in 95% ethanol for 24 hours, followed by acetone for 24 hours. Tibias were stained with 0.015% Alcian blue, 0.05% alizarin red and 5% acetic acid in 70% ethanol. The stained tibias were stored in glycerol/ethanol (1:1). Images were taken with a Nikon SMZ1500 dissecting microscope with a Photometrics Coolsnap camera using ImageMaster 5.0 software. At least 3 independent experiments containing 4-6 tibias per treatment (n) were used.

2.2.5 Histology and immunohistochemistry (IHC)

Organ culture and isolated P0 tibias were fixed in 4% paraformaldehyde overnight and decalcified with 0.1M EDTA/PBS at room temperature before paraffin embedding and sectioning at the Robarts Research Institute Molecular Pathology Core Facility (London, ON). Five µm sections were dewaxed in xylene followed by a graded series of ethanol washes (100% x 2, 95% x 1, 70% x 1). Sections were stained with either H&E or Safranin O/Fast green (39, 40, 43, 44). For IHC, sections were incubated in 3% H2O2 for
15 min at room temperature, followed by antigen retrieval by incubation in either 10 mM sodium citrate at 95 °C for 30 min or 0.1% triton X for 10 min, followed by blocking with 5% goat serum in PBS. Sections were incubated with primary antibody overnight at 4 °C, washed (PBS x 4), and secondary antibody was applied according to manufacturers’ recommendations. For detection, DAB substrate was used and counterstained with Methyl Blue. All images were taken with a Leica DME microscope with a Qimaging Mircropublishes 5.0 RTV camera using QCapture Pro 5.1 software.

Quantification of IHC was conducted depending on the target protein. For quantification of PCNA IHC, the positively stained (brown) and total nuclei were counted to determine the fraction of PCNA positive cells in the growth plate. The quantification of p57 protein in the growth plate was conducted by measuring the length (proximal to distal) of the prehypertrophic zone where darkly stained (brown) nuclei are present.

2.2.6 Western blot analyses

Fresh calvaria, cartilage from the epiphyseal ends of long bones (humerus, femur, tibia), and organs were dissected from P0 mice in cold Puck’s solution A (PSA) (39, 45). Samples were flash frozen in RIPA buffer and stored at -20 °C overnight, then homogenized, sonicated and centrifuged. Total protein content was determined; 25-35 µg total protein (depending on protein yield) was loaded per lane in precast NuPAGE Novex Midi Tris-Acetate Gels and separated using the XCell Surelock Mini-cell (Invitrogen) system. Gels were blotted using XCell II Blot Module (Invitrogen) as per manufacturer’s instructions. Blots were blocked in 5% BSA TBST solution for 1 hour, and then probed with primary antibody overnight at 4 °C. After washing (TBST), membranes were incubated with appropriate secondary antibody (hrp-conjugated) for one hour at room temperature, and the resultant signal was detected using the ECL detection system (Amersham). Representative blots from at least 3 independent pairs of littermates are shown. Quantitative densitometry analysis was conducted using a ChemiImager 5500 system, subtracting background and normalizing to β-actin loading control signal. Densitometry results were converted relative to control, allowing comparison between blots and compared statistically by t-test analysis.
2.2.7 Statistical analysis

All data were collected from at least 3 independent organ culture trial or pairs of littermates. Data was expressed as mean ±SE, and p values under 0.05 were considered significant. For general measurements and comparisons between two groups of data, statistical significance was determined by unpaired t-test comparing control to treated (SB86) or control to cartilage specific deletion of GSK-3β (KO) littermates using GraphPad Prism 3.00 for Windows. Western blot densitometry data were normalized to controls and compared by t-test. Comparison of multiple treatments was done using one-way Anova (normal distribution analyzed) and a Tukey post-test.

2.3 Results

2.3.1 GSK-3 expression in the growth plate

To determine the expression patterns of both GSK-3 proteins in the growth plate in vivo, we performed immunohistochemistry (IHC) on wild type P0 mouse tibia. GSK-3β was expressed at low levels in chondrocytes at the articular surface and hardly detectable by IHC in resting and proliferating chondrocytes, but strongly expressed in prehypertrophic and hypertrophic chondrocytes (Fig. 2.1). GSK-3β in the hypertrophic chondrocytes appears both cytoplasmic and nuclear with the highest concentration being nuclear. In contrast, GSK-3α showed strong expression throughout the growth plate and appeared to be more cytoplasmic than β. Both proteins were also expressed in the perichondrium where GSK-3β appeared confined to the layer of cells closest to hypertrophic chondrocytes and GSK-3α was again more uniformly expressed through the cells of the perichondrium.

2.3.2 GSK-3 inhibition increases bone growth and affects growth plate morphology

To determine the role that GSK-3 plays in endochondral bone growth, e15.5 tibia organ culture were cultured for 6 days with the pharmacological GSK-3 inhibitor SB415286 (SB86; 10 μM) that inhibits both GSK-3 proteins (42). Longitudinal growth over this
Fig. 1. GSK-3β

- Resting
- Proliferating
- Hypertrophic

GSK-3α

Perichondrium
Figure 2.1 (9)- Expression of GSK-3 proteins in the growth plate

Expression of GSK-3α and β proteins in the growth plate of wildtype P0 tibia was analyzed by IHC. While GSK-3β expression was detected in chondrocytes of the articular surface (Arrow) and in particular in prehypertrophic and hypertrophic chondrocytes, GSK-3α is expressed in all zones of the growth plate. Both proteins are also expressed in the perichondrium (P), although GSK-3β was restricted to the cells closest to hypertrophic chondrocytes (H) and GSK-3α was again uniformly expressed throughout the cells of the perichondrium.
time was compared to controls treated with DMSO. Tibia treated with SB86 grew 31% more than controls over the 6 days of organ culture (Fig 2.2 A, B). A similar trend, though not statistically significant with a completed n of 3, was also observed using another GSK-3 inhibitor, 10µM SB216763. SB86 was used exclusively for the remainder of the experiments. Interestingly, this increase in bone growth did not affect the overall length of the growth plate of the treated tibia (Fig 2.2 C, D). However, inhibition of GSK-3 did affect the organization and relative lengths of the zones within the growth plate (Fig 2.2 E, F). The division between the resting and proliferating zones was much less distinct in the SB86-treated bones and therefore the zone measurements combined both resting and proliferating zones for accuracy of measurements. The resting and proliferating zones of the treated tibia were 13% longer than the controls (Fig 2.2 E), whereas the length of hypertrophic zone was significantly decreased by 57% (Fig 2.2 F). Since the inhibition of GSK-3 caused increased tibia growth but the total growth plate length was not increased, we examined the mineralized portion of the tibia (Fig 2.2 G). The mineralized portion of the tibia was found to be significantly longer as a ratio of total tibia length. These results together suggest that inhibition of GSK-3 promotes long bone longitudinal growth by increasing bone formation.

2.3.3 GSK-3 inhibition rescues most effects of PI3K inhibition in organ culture

GSK-3 activity is regulated through N-terminal phosphorylation by the insulin/PI3K/Akt pathway (12, 46) as well the IGF/PI3K/Akt pathway that is an important regulator of chondrocyte physiology (15, 47). We previously showed that PI3K inhibition decreased bone growth and the length of the proliferating and hypertrophic zones (15). Given that PI3K activates PKB/Akt that in turn phosphorylates and inhibits GSK-3, inhibition of PI3K should activate GSK-3 and therefore have opposite effects as inhibiting GSK-3. Furthermore, if inactivation of GSK-3 is one of the main effectors of PI3K signaling in endochondral bone growth, then GSK-3 inhibition should rescue the effects of PI3K inhibition, at least in part. To explore interactions between PI3K/Akt and GSK-3, the effects of treatments with a PI3K inhibitor (10 µM LY 294002, LY) in combination with
Figure 2.2 (10) - Inhibition of GSK-3 increases bone growth and changes growth plate organization

E15.5 mouse tibiae were cultured in organ culture (OC) for 6 days with a pharmacological inhibitor of GSK-3 (10µM SB415286; SB86) or DMSO (Cont). (A) Representative picture of tibiae stained with Alizarin Red (Bone) and Alcian Blue (Cartilage). (B) Quantification of growth (mm) of the tibia during the 6 days of treatment. (C) H&E-stained paraffin sections of organ culture tibiae comparing morphology and growth plate zones, e.g. resting and proliferating zones (R/P) and hypertrophic zone (H). (D) Length (mm) of the proximal tibia growth plate in control and SB86-treated tibiae. (E) Resting and proliferating zone length (mm) in control and GSK-3 inhibited organ culture. (F) Hypertrophic zone length (mm) in control and GSK-3 inhibited organ culture. (G) Quantification of the proportion of mineralized bone relative to the whole tibia length in control and GSK-3-inhibited organ culture. All data were compared using t-test analysis, significant differences are denoted by asterisk (n > 8, *: p < 0.05).
the GSK-3 inhibitor (10 μM SB415286, SB86) on tibia organ cultures were examined (Fig 2.3).

To confirm that the inhibition of PI3K would indeed affect the phosphorylation of GSK-3 in our tibia organ culture model, IHC analyses was conducted using antibodies specific to phosphorylated GSK-3β (Fig 2.3A upper) or to phosphorylated forms of both GSK-3 proteins (Fig 2.3 A lower). These data shows that phosphorylation for GSK-3β was consistently decreased with LY treatment through the pre-hypertrophic/hypertrophic zones where GSK-3β is primarily expressed in the growth plate (Fig 2.3 A upper). The IHC detecting both phospho-GSK-3α and β demonstrated a decrease in the GSK-3 phosphorylation through the resting and proliferating zones but a less significant change in the pre-hypertrophic zone (Fig 2.3 A lower). This suggests that GSK-3α, which is more widely expressed in the growth plate, is the predominate GSK-3 form targeted by inhibition of the PI3K pathway through the resting and proliferating zones, whereas GSK-3β is the main target the prehypertrophic zone.

Consistent with our earlier studies, SB86 (Fig 2.2) increased and LY (15) decreased tibia growth in culture (Fig 2.3 B, C). The effect of the PI3K inhibitor was partially recovered by the combination with the GSK-3 inhibitor (LY + SB86) (Fig 2.3 B, C). None of the treatments had significant effects on the total length of the growth plates. Similar to the bone growth, the combination of the inhibitors was able to partially recover the effect of PI3K inhibition on the length of the resting/proliferative zone (Fig 2.3 D), while both inhibitors individually and in combination reduced the length of the hypertrophic zone (Fig 2.3 E).

2.3.4 Chondrocyte-specific ablation of GSK-3β in vivo

To determine if the ex vivo tibia organ cultures results would translate into an in vivo model and to address the roles of individual GSK-3 proteins, we generated mice with cartilage-specific loss of GSK-3β. Mice homozygote for the floxed alleles of GSK-3β (Gsk3b^fl/fl) (16, 38) were crossed with mice heterozygote for the floxed allele and expressing cre recombinase gene under control of the mouse collagen II (Col2a1) promoter (Gsk3b^fl/wt cre^+) (39, 48). This breeding scheme produced cartilage-specific
Figure 2.3 (11) Interaction of PI3K and GSK-3 signaling in organ culture

Tibia organ cultures (e15.5) were treated with GSK-3 inhibitor (10µM SB415286 (SB86)), PI3K inhibitor (10 µM LY 294002 (LY)), a combination of both inhibitors (10µM SB415286 + 10 µM LY 294002 (SB86 + LY)), or DMSO (Cont) for six days. (A) IHC analyses of GSK-3 phosphorylation using phospho-specific antibodies specific for GSK-3β (upper panel) or both GSK-3α and β (lower panel). (B) Representative picture of tibiae after six days of culture stained with Alizarin Red (Bone) and Alcian Blue (Cartilage). (C) Quantification of longitudinal growth of tibiae (mm) over 6 days of culture. (D) Resting and proliferating zone length (µm) of bones treated with the various inhibitors. (E) Hypertrophic zone length (µm) of bones treated with the various inhibitors. All data were compared using one-way ANOVA with a Tukey post-test analysis, significant differences are denoted by asterisk (n = 3, *: p < 0.05).
GSK-3β deletion in (Gsk3b<sup>fl/fl cre</sup>+) referred to as knockout mice (KO), heterozygous mice (Gsk3b<sup>fl/wt cre</sup>−), as well as control (Cont) littermates (Gsk3b<sup>fl/fl cre</sup>− or Gsk3b<sup>fl/wt cre</sup>). Efficiency of GSK-3β deletion was determined through Western blot analysis and immunohistochemistry (Fig 2.4). Long bone growth plate extracts from P0 mice demonstrated an 80% reduction in GSK-3β protein in KO cartilage (Fig 2.4 A, B). Immunohistochemistry (IHC) of P0 tibia paraffin section demonstrated strong expression of GSK-3β in the prehypertrophic and hypertrophic zones of control mice with virtually a complete loss of signal in KO littermates (Fig 2.4 C). GSK-3β protein levels were explored in many tissues to evaluate the specificity of the deletion (Fig 2.4 D, E). Calvaria tissue samples showed that GSK-3β expression was not affected in bone tissue (Fig 2.4 D). No loss of GSK-3β protein was observed in any other tissues of KO mice (Fig 2.4 E).

The cartilage specific GSK-3β KO mice did not display an observable skeletal phenotype. The size and weight of KO mice were similar to those of control sex-matched littermates throughout their life, from birth to 1 year (data not shown). The length of the tibiae at birth P0 (Fig 2.4 F) and at P21 were not significantly different from control mice. The growth plate zones were also not visually affected upon cartilage-specific GSK-3β deletion (Fig 2.4 G).

### 2.3.5 GSK-3 inhibition increases chondrocyte proliferation

To determine why ex vivo inhibition of GSK-3 effects bone growth whereas in vivo GSK-3β deletion had no observable effect, the cellular mechanisms controlling growth plate dynamics were explored. Since the rate at which chondrocytes cycle through the growth plate stages determines both zone morphology and bone growth, chondrocyte proliferation was assessed through IHC analyses using Proliferating Cell Nuclear Antigen (PCNA) antibodies (Fig 2.5). The PCNA IHC was quantified as the fraction of positively stained cells per total number of cells. GSK-3 inhibition (SB86) did increase the amount of actively proliferating cells in the tibia organ culture almost twofold (Fig 2.5 A, B). Proliferation was not significantly decreased below the control levels upon treatment with the PI3K inhibitor (LY) while treatment with both inhibitors increased proliferation.
Figure 2.4 (12) - Cartilage-specific deletion of GSK-3β.

Cartilage-specific GSK-3β KO mice carrying 2 alleles of the floxed Gsk3b gene and expressing cre recombinase under the control of the collagen II (Col2a1) promoter (KO) were created and compared to the control littermates (Cont). (A) Western blot analyses using GSK-3β antibodies conducted on growth plate (GP) protein extracts from new born littermate mice of Gsk3b^fl/fl cre^+ (KO) and control (Gsk3b^fl/fl cre^- or Gsk3b^fl/wt cre^-) (Cont) genotype. (B) Densitometric quantification of GSK-3β protein in P0 growth plate extracts. (C) IHC using GSK-3β antibodies on paraffin sections of P0 tibia. Black box in top image indicates the location of the lower higher magnification images. (D) Western blot analyses of GSK-3β protein in calvarial tissue (Calv). (E) Western blot analyses of GSK-3β protein in heart, brain, and kidney protein samples of control (C) and knockout mice (KO). (F) Tibia length (mm) measurements from P0 control and KO mice. (G) Paraffin sections of P0 tibiae from control and KO mice stained with Safranin O. All Western blot analyses used β–actin as a loading control. Quantification used t-test analyses where significance was denoted with an asterisk (n > 3, *: p < 0.05).
Fig. 5.

A. Organ Culture PCNA

B. Organ Culture PCNA

C. GSK-3β KO PCNA

D. GSK-3β KO PCNA

Bar graphs showing the percentage of positive cells in control (Cont), SB86, LY, and LY + SB86 conditions. The graph on the left illustrates the effect of GSK-3β KO on PCNA expression in resting and proliferating states.
Figure 2.5 (13) - GSK-3 inhibition increases proliferation in organ culture while GSK-3β deletion in cartilage has no effect on proliferation in vivo.

Proliferating Cell Nuclear Antigen (PCNA) was utilized to stain proliferating cells in both tibia organ culture (OC) experiments and cartilage-specific GSK-3β KO mice. (A) IHC analyses of PCNA on paraffin section of tibia organ cultures treated with GSK-3 inhibitor (10µM SB415286 (SB86)), PI3K inhibitor (10 µM LY 294002 (LY)), a combination of both inhibitors (10µM SB415286 + 10 µM LY 294002 (SB86 + LY)), or DMSO (Cont). (B) Quantification of the PCNA staining from (A) as percentage of stained cells to total cells. Results were statistically compared using a one-way ANOVA with a Tukey post-test analyses, significance is denoted by letter asterisks (n = 3, *: p < 0.05). (C) PCNA IHC analyses of P0 tibia from cartilage-specific GSK-3β KO mice (KO) and control littermates (Cont). (D) PCNA staining quantified as fraction of positive stained cells. Results were compared by t-test. (n = 3, *: p > 0.05)
significantly over the LY treatment, similar to the effect of GSK-3 inhibition only (Fig 2.5 B). Similar data was obtained using BrdU labelling and detection (Data not shown). In contrast, no difference in the fraction of PCNA-positive chondrocytes was observed upon cartilage-specific GSK-3β deletion (Fig 2.5 C, D).

2.3.6 Prehypertrophic cell cycle exit increased upon GSK-3 inhibition

Hypertrophic chondrocyte differentiation is initiated by cell cycle exit, giving rise to prehypertrophic chondrocytes (49, 50). The cyclin-dependent kinase inhibitor p57 is both a marker of prehypertrophic chondrocytes and promotes cell cycle exit in the growth plate (49-54). In tibia organ culture, inhibition of GSK-3 or dual treatments of GSK-3 and PI3K inhibitors greatly increased the zone of p57 protein expression in the prehypertrophic zone (Fig 2.6 A black arrows insets) while the addition of the PI3K inhibitor did reduce this zone, although not statistically significantly (Fig 2.6 A, B). This p57 positive staining was localized to the cell nucleus in the prehypertrophic zone (Fig 2.6 A far right inset). In cartilage-specific GSK-3β KO mice, p57 protein expression was similar to that of control littermates (Fig 2.6 C), and quantification did not show any significant change in the length of the p57-positive zone (data not shown).

2.3.7 β-catenin expression in prehypertrophic chondrocytes is upregulated upon inhibition of GSK-3

Arguably the most studied downstream target of GSK-3 is the transcription factor β-catenin, a target of the canonical Wnt pathway that is a central regulator of skeletal development (7, 55-59). An increase in the amount to β-catenin protein was observed specifically throughout the prehypertrophic/hypertrophic zone of tibiae in organ culture treated with the GSK-3 inhibitor (Fig 2.7 A). IHC for β-catenin was also performed on the other two treatments discussed above, LY and SB86+LY, however PI3K inhibition yielded no change and dual inhibition results were inconclusive (data not shown). Western Blot showed no significant change in β-catenin protein levels in the growth plate of cartilage-specific GSK-3β KO mice, although levels consistently appeared increased in
Figure 2.6 (14) - GSK-3 regulates p57 expression in the prehypertrophic zone in vitro while cartilage-specific GSK-3β deletion does not affect p57 expression in vivo.

(A) IHC analyses of p57 expression in paraffin sections of tibia organ culture treated with GSK-3 inhibitor (10 µM SB415286 (SB86)), PI3K inhibitor (10 µM LY 294002 (LY)), a combination of both inhibitors (10µM SB415286 + 10 µM LY 294002 (SB86 + LY)), or DMSO (Cont). Black boxed insets are higher magnification images depicting the zones of p57 staining, black arrows. Far right image is a higher magnification (boxed area) of staining to determine cellular localization. (B) Quantification of the length (µm) of the zone of p57 staining observed in (A). Data was analyzed by one-way ANOVA with a Tukey post-test with significance denoted by asterisk (n > 3, *: p < 0.01). (C) IHC analysis of p57 expression in paraffin sections of P0 tibiae of cartilage-specific GSK-3β KO mice compared to littermate controls (Cont).
Figure 2.7 (15) GSK-3β regulates β–catenin expression in the prehypertrophic zone.

(A) Sections of tibia organ cultures treated with a GSK-3 inhibitor (10µM SB415286 (SB86)) or DMSO (Cont) were analyzed by IHC using β–catenin antibodies. Black brackets indicate the zone of β-catenin stained cells in the prehypertrophic zone. (B) Western blot analyses of P0 growth plate protein extracts from cartilage-specific GSK-3β KO mice (KO) and control (Cont) littermates probed with β–catenin antibodies, with β-actin (Actin) as loading control. (C) Localization of β–catenin protein was conducted by IHC using β–catenin antibodies on paraffin tibia sections from mice described in (B).
mutant cartilage (Fig 2.7 B). However, IHC suggested that β-catenin levels are increased is specific subsets of chondrocytes in cartilage-specific GSK-3β KO mice, in particular prehypertrophic chondrocytes and the articular surface (Fig 2.7 C).

2.3.8 Upregulation of GSK-3α protein in response to GSK-3β deletion in vivo

The two forms of GSK-3, α and β, play both overlapping and distinct roles (16, 17, 28, 29, 31). GSK-3α levels and overall phosphorylation of the GSK-3 proteins were examined to understand the apparent lack of phenotype in the cartilage-specific GSK-3β KO mice (Fig 2.8). Surprisingly, Western blotting demonstrated an approximately two-fold increase in GSK-3α protein in KO cartilage (Fig 2.8 A, B). IHC further supported these data by showing an increase in GSK-3α protein throughout the pre-hypertrophic and hypertrophic area as well as the resting zone and articular surface (Fig 2.8 C). To a lesser extent an increase in GSK-3α protein was observed in the proliferating zone (Fig 2.8 C). There was no obvious change in the total phosphorylation of the GSK-3 proteins in all zones (Fig 2.8 D), providing further evidence that upregulation of GSK-3α compensates for the loss of GSK-3β in cartilage.

2.4 Discussion

This study contributes important and novel data to our understanding of both skeletal development and GSK-3 signaling. Our results demonstrate profound effects of GSK-3 inhibition on bone growth in a tibia organ culture system, which is contrasted by minimal phenotypes observed upon cartilage-specific deletion of GSK-3β. This absence of phenotype is likely due to compensatory upregulation of GSK-3α expression, a mechanism that has, to our knowledge, not been reported for any other tissue or context.

Our tibia organ culture experiments show that inhibition of GSK-3 results in increased longitudinal growth of endochondral bones. This was likely caused, at least in part, by increased proliferation of chondrocytes in the resting and proliferating zones. The shortened hypertrophic zone resulting from GSK-3 inhibition appears to be
Fig. 8.

A. Cont GP KO

B. Growth Plate GSK-3α

C. Cont GSK-3α KO

D. pGSK-3α + β

Relative Densitometry

n=3

p<0.05

Articular

Resting

Proliferating

Hypertrophic

Resting

Proliferating

Hypertrophic
Figure 2.7 (16) - *In vivo* deletion of GSK-3β causes upregulation of GSK-3α protein in chondrocytes.

(A) Western blot analyses of GSK-3α protein in growth plate protein extracts of cartilage-specific GSK-3β KO and control mice, with β-actin as loading control. (B) Densitometric quantification of GSK-3α Western blot analyses (A); statistical analysis was conducted using t-test, significance is indicated by asterisk (*) (n = 3, *: p < 0.05). (C) IHC analyses using GSK-3α antibodies on paraffin sections of PO KO and control tibiae. (D) IHC analysis using antibodies detecting the phosphorylated state of both GSK-3 forms, α and β.
counterintuitive to the increase in growth observed by us. However, the larger proportion of PCNA-labelled chondrocytes, the increase in p57 staining and the relative increase in the length of the mineralized zone all suggest that all processes in the growth plate (proliferation, differentiation and replacement of cartilage by bone) occur at a faster rate upon GSK-3 inhibition, thus resulting in increased bone growth. The reduced length of the hypertrophic zone would therefore not be due to delayed differentiation, but rather to faster turnover of hypertrophic cartilage to bone.

Our observations of increased chondrocyte proliferation and accelerated bone maturation in response to GSK-3 inhibition are opposite to findings from Naski and colleagues who showed that a different pharmacological GSK-3 inhibitor reduced chondrocyte proliferation and differentiation in a metatarsal organ culture system (60). These differences might be due to the nature and/or concentration of the inhibitors, the duration of treatment or the identity of the skeletal elements investigated, suggesting that the effects of GSK-3 in cartilage might be context-dependent. However, our data showing that GSK3 inhibition promotes endochondral bone growth – which is unlikely to occur when both proliferation and hypertrophy of chondrocytes are inhibited – are supported by a recent in vivo study where loss of one allele of the Gsk3b gene rescues the dwarfism of mice deficient for cGMP-dependent kinase II (cGKII), another upstream inhibitor of GSK-3 (24). cGKII is a key mediator of the anabolic effects of C-type natriuretic peptide on endochondral bone growth (61). These data by Kawasaki et al. (24) show that inhibition of GSK-3 activity is required for the anabolic effects of cGKII in bone, an effect we mimic by pharmacological inhibition of GSK-3.

Our data also provide insights into the relationship between PI3K and GSK-3 signaling in cartilage. PI3K inhibition resulted in decreased phosphorylation (indicating increased activity) of GSK-3. In agreement with these data, pharmacological inhibition of GSK-3 rescued many of the effects of the PI3K inhibitor, in particular on bone growth. However, this was not true for the length of the hypertrophic zone, which was reduced by both inhibitors individually or in combination. However, these effects on the hypertrophic zone could be due to the different mechanisms, since PI3K inhibition appears to delay chondrocyte hypertrophy and the associated bone growth ((15) (reviewed in 62)), while
our data presented here suggest that GSK-3 inhibition accelerates the replacement of hypertrophic cartilage by mineralized tissue. Furthermore, the similar effects of both inhibitors on the hypertrophic zone reflect the fact that PI3K also acts through other downstream effectors in addition to GSK-3 (63), while GSK-3 is also involved in the response to signals not mediated by PI3K, such as Wnts.

Given the significant impact of GSK-3 inhibition in organ culture, the absence of a clear phenotype in cartilage-specific Gsk3b KO mice was surprising. However, our subsequent molecular analyses revealed the putative explanation. First, the expression of GSK-3β in cartilage is quite restricted, at least in comparison to the more ubiquitously expressed GSK-3α. These different expression patterns were also shown at the mRNA level in our earlier microarray studies on microdissected mouse growth plates (64). More importantly, GSK-3α expression was upregulated markedly in all zones of the growth plates of Gsk3b-mutant mice, even where very low levels of GSK-3β were seen in wild type mice (such as in the proliferative zone). These data suggest that GSK-3α is able to compensate for most functions of GSK-3β in chondrocytes. However, our results cannot exclude alternative explanations for the lack of a phenotype in our KO mice; analyses of double KO mice for both Gsk3 genes will be required to firmly establish redundant functions of these two genes.

The molecular basis of the increase in GSK-3α levels in our mutants is currently unknown. Since GSK-3β regulates the stability of many other proteins (65), it is tempting to speculate that loss of GSK-3β leads to stabilization of GSK-3α protein. However, to our knowledge no such cross-talk between the two GSK-3 proteins has been described. Alternatively, it is plausible that increased GSK-3α expression in our KO mice is due to increased transcription, mRNA stability and/or translation. Future studies will need to address the mechanisms involved.

The one exception to compensation appeared to be upregulation of β-catenin protein levels in specific chondrocytes of cartilage-specific Gsk3b KO mice, suggesting that GSK-3α is not able to fully substitute for GSK-3β function in all chondrocytes. Since this effect was only seen in a few chondrocytes, it was not reflected in Western blot analyses
of extracts from the entire cartilage, but it was very reproducible in immunohistochemical analyses of tissue sections. Notably, increased staining for β-catenin in mutant mice was seen in exactly those cells that express detectable levels of GSK-3β in wild type mice (e.g. articular and prehypertrophic/hypertrophic chondrocytes), suggesting that GSK-3α can not fully compensate for this aspect of GSK-3β function in these cells. The reason for this inability is unclear, but might be related to the particular levels of expression of the two GSK-3 proteins in specific chondrocyte populations. Analyses of double mutants for both Gsk3 genes will provide further insight into this unexpected finding. While chondrocyte function is very sensitive to both supra- and superphysiological levels of β-catenin (56, 57), the moderate change in expression observed here appears to be insufficient to induce changes in cartilage development and bone growth, at least over the time frame evaluated here. It will be interesting to examine in the future whether increased β-catenin or other aspects of cartilage-specific loss of GSK-3β alters the susceptibility to cartilage degeneration in osteoarthritis.

In closing, we believe that this study contributes important and novel information to our understanding of the complicated relationship between the two GSK-3 proteins as well as to our understanding of the signaling pathways controlling endochondral bone development. Additional studies, such as the simultaneous inactivation of both Gsk3 genes in cartilage, will further elucidate the role of these key signaling molecules in the physiology and pathophysiology of cartilage.

2.5 References


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Chapter 3

3  Skeletal-specific deletion of Glycogen Synthase Kinase 3 beta (GSK-3β) delays postnatal growth and skeletal development

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3.1 Introduction

The bones of the skeleton are formed through two different processes, endochondral and intramembranous ossification (reviewed in (1-3)). Most of the skeleton forms through endochondral ossification in which a cartilage scaffold is produced by chondrocytes before it is converted to bone, produced by osteoblasts. Growth plate chondrocytes are found at either end of long bones and arranged in distinct layers of resting, proliferating and hypertrophic cells, differing in gene expression patterns, rate of cell cycle progression, and cell morphology (reviewed in (3-6)). Surrounding the growth plate is the perichondrium, containing pre-osteoblasts that differentiate into mature osteoblasts to form the bone collar or cortical bone through intramembranous ossification (reviewed in (7,8)). Intramembranous ossification also forms the calvariae of the skull and occurs when bone is formed without a cartilage template. Thus, the skeleton is a large and complex component of the body requiring precise autocrine, paracrine and endocrine signaling to form properly.

Glycogen Synthase Kinase-3 (GSK-3) is an ubiquitous cellular regulator that functions as a “brake” on many anabolic pathways, such as the Wnt/β-catenin and insulin-activated pathways. GSK-3 is an unusual protein kinase due to its high level of activity in resting tissues, its inhibition upon cellular stimulation, and its role as a negative regulator of the activity of many substrates. Mammals have two GSK-3 genes that encode GSK-3α and GSK-3β with molecular masses of 51kDa and 47kDa, respectively (reviewed in (7-9)). Inhibition of GSK-3α and β can occur through at least two mechanisms: direct phosphorylation (Ser21 and Ser9 of GSK-3α and β, respectively) as occurs through the
insulin/phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway (10), or through disruption/sequestration of the protein complex involved in canonical Wnt/β-catenin signaling (9). The specific roles of GSK-3α and/or β in regulation of the various signaling pathways is unclear since they appear functionally redundant in some studies (11,12), but differential and tissue-specific roles for the two forms have also been demonstrated (13-17). Moreover, the relative roles of GSK-3 can be dependent on the genetic background or strain of mice examined (18).

Other groups have studied the phenotypic consequences of manipulating Gsk3b expression on the skeleton (19-21). Germline homozygous deletion of Gsk3b causes a variable phenotype depending on genetic background and can result in embryonic lethality (19) or survival to P0 with cleft palate, bifid sternum and delayed ossification of the sternum, skull, ear bones, and cranial base (20). In contrast, heterozygous deletion of Gsk3b caused increased ossification, clavicle abnormalities and increased bone resorption (21). It would appear that these opposing skeletal phenotypes (e.g. delayed versus increased ossification) are Gsk3b dosage-dependent effects; however, all these phenotypes are based on germline loss of Gsk3b. Consequently, it is unclear whether these skeletal phenotypes are cell- or tissue-autonomous.

We previously generated mice in which GSK-3β was specifically deleted in chondrocytes and compared their in vivo phenotype with the effects of pharmacological inhibitors of GSK-3 on ex vivo tibia organ cultures (22). The ex vivo studies demonstrated that inhibition of GSK-3 (both α and β) increased bone growth. However, the characterization of cartilage-specific GSK-3β deficient mice did not reveal any phenotype with the exception of an increase in GSK-3α protein expression. This lack of phenotype could
have been due to compensation by GSK-3α; however, these observations suggest that skeletal phenotypes in the global GSK-3β KO mice (20) are likely due to functions of GSK-3β in other skeletal lineages such as osteoblasts. To address this possibility, we created mice in which GSK-3β was inactivated in early differentiating skeletal cells and osteoblasts.

3.2 Materials and methods

3.2.1 Materials

The following antibodies were utilized in this study: Cre ab24608, Sox9 ab3697 (Abcam, Cambridge, MA); actin A5441, Runx2 R9403 (Sigma Chemical Co., St. Louis, MO); goat anti-rabbit hrp sc-2004, donkey anti-goat hrp sc-2020, goat anti-mouse hrp sc-2005, Rankl sc-9072, p57/Kipp2 sc-8298 (Santa Cruz Biotechnology, Santa Cruz, CA); GSK-3β #9315, pGSK-3β #9336, GSK-3α #9338, PCNA #2586, cleaved caspase 3 #9661, β-catenin #9562 (Cell Signaling Technology, Danvers, MA). General chemicals and supplies were purchased from Sigma and VWR (Radnor, PA), organ culture reagents were from Invitrogen (Carlsbad, CA).

3.2.2 Mouse breeding and genotyping

Mice homozygous for floxed Gsk3b alleles (Gsk3b<sup>0/0</sup>) have been described previously (17,22-24) and were crossed with mice expressing cre recombinase under the control of an osteoblast-specific 3.6kb fragment of the rat Col1a1 promoter (25,26). Male mice heterozygous for the floxed Gsk3b allele and expressing cre under control of the 3.6 kb Col1a1 promoter were backcrossed with homozygous Gsk3b floxed female mice. The offspring from these crosses were analyzed. Mice were exposed to a 12 hr light-dark
cycle and fed tap water and regular chow *ad libitum*. All procedures involving animals were approved by the University of Western Ontario Animal Care and Use Committee. PCR genotyping was performed from ear notch DNA using primers 5’-GGGGCAACCTTAATTTCAATT-3’ (forward) and 5’-TCTGGGCTATAGCTATCTAGTAACG-3’ (reverse) for GSK-3β for 30 cycles of 96 °C 55sec, 56.5 °C 45 sec, 68 °C 2:45min to amplify. The cre transgene was detected using the primers 5’-CACACTGTGTAGTGCTTCGT-3’ (forward) and 5’-CCTCCAAACCATCCAAGAT-3’ (reverse) using 40 cycles of 95 °C 45sec, 58 °C 30sec, 72 °C 1min. Cartilage-specific KO mice were bred and analyzed as described (22).

3.2.3 Skeletal staining
Skeletal staining was performed as described previously (22,24,27,28). Briefly, the skin and organs were removed and the resulting skeletal preparation was dehydrated in 95% ethanol for 24 hours, followed by acetone for 24 hours. Skeletons were stained with 0.015% Alcian blue, 0.05% alizarin red and 5% acetic acid in 70% ethanol. The length of staining depended on the age of specimen when harvested, and was up to 4 days for 21-day-old mice. Skeletons were cleared in 2% KOH overnight, 1% KOH for several days and 0.5% KOH for approximately 1 week (varying with age of specimen). The stained skeletons were stored in glycerol/ethanol (1:1). Images were captured with a Nikon SMZ1500 dissecting microscope with a Photometrics Coolsnap camera using ImageMaster 5.0 software. At least 3 independent littermate pairs (control and knockout) were examined per time point. Representative examples of each skeletal abnormality are shown.
3.2.4 Histology and immunohistochemistry (IHC)

Freshly dissected tibia from both P0 and P21 mice were fixed in 4% paraformaldehyde overnight and decalcified with 0.1M EDTA/PBS at room temperature before embedding and sectioning at the Robarts Research Institute Molecular Pathology Core Facility (London, ON). 5µm sections were dewaxed in xylene followed by a graded series of alcohol washes, 100% x2, 95% x1, 70% x1. Sections were stained with either Picrosirius Red, Safranin O/Fast green, or Von Kossa/Toluidine blue. Measurements of trabecular length from the hypertrophic/bone interface to the average end of the trabecular bone in the middle of the tibia section were performed using Qcapture software. IHC sections were incubated in 3% H₂O₂ for 15 min at room temperature, followed by incubation in 10 mM sodium citrate at 95°C for 30 min and blocking with 5% goat serum in PBS (27-30). Sections were incubated with primary antibody overnight at 4°C, washed 4 times in PBS, and secondary antibody was applied according to manufacturers’ recommendations. For detection, DAB substrate was used and counterstained with methyl blue. All images were taken with a Leica DME microscope with a Qimaging Micropublisher 5.0 RTV camera using QCapture Pro 5.1 software.

Alternatively, bones were not decalcified and shipped to The Centre for Bone and Periodontal Research (Montreal, QC, Canada) for plastic embedding, sectioning (5µm) and staining with Von Kossa/Toluidine Blue.

Sections were examined using kits for TUNEL (Calbiochem) and TRAP (Sigma), as per manufacturers’ instructions with minor optimizations. TRAP staining was performed overnight at 4°C. Representative stains, IHC, and labelling from at least three independent pairs of littermates are shown.
3.2.5 Western blot analyses

Fresh calvaria and cartilage from long bones were dissected from P0 mice in cold Puck’s solution A (PSA) (22,24,27,31). Samples were flash frozen in RIPA buffer and stored at -20 °C overnight, then homogenized, sonicated and centrifuged. After total protein content was determined, 25-35µg total protein was loaded per lane into precast NuPAGE Novex Midi Tris-Acetate Gels and separated using the XCell Surelock Mini-cell (Invitrogen) system. Gels were blotted using XCell II Blot Module (Invitrogen) as per manufacturer’s instructions. Blots were blocked in 5% BSA TBST solution for 1 hour, and then probed with primary antibody overnight at 4 °C. Appropriate secondary antibody (HRP-conjugated) was incubated for one hour at room temperature, and the resultant signal was detected using the ECL detection system (Amersham). Representative blots from at least 3 independent pairs of littermates are shown. Quantitative densitometry analysis was conducted using a ChemiImager 5500 system subtracting background and normalizing to the β-actin loading control signal. Densitometry results were converted relative to control, allowing comparison between blots by t-test analysis.

3.2.6 Serum analysis

Serum readings were performed using a Millipore Multiplex system for OPG (Cat No. MBN1A-41K), Rankl (Cat No. MBN-41K-1RANKL), IGF-1 (Cat No. RMIGF187K), and GH (Cat No. EZRMGH) in the Screening Lab for Immune Disorders, St Joseph Hospital, London, Ontario, Canada.
3.2.7 Micro-CT

3.2.7.1 Image acquisition

Images of isolated p21 humeri were acquired using a micro-computed tomography (micro-CT) system (eXplore Locus SP, GE Healthcare Biosciences, London, Ontario, Canada). The scanning protocol included 900 views acquired over 2.5 hours, using an x-ray tube potential of 80kVp and tube current of 80 µA. Images were reconstructed with an isotropic resolution of 26 µm. In addition to the specimen scans, a separate calibration phantom containing air, water and hydroxyapatite mimic (SB3, Gammex RMI, Middleton, WI, USA) was scanned and used to convert the original reconstructions into Hounsfield Units.

3.2.7.2 Cortical bone analyses

Cortical bone analyses was performed using microCT images of humeri using MicroView software. A mid-diaphyseal defined volume of interest (VOI) (1.7 mm x 1.7 mm x 0.2 mm) was defined by limiting the VOI to the appearance of the distal end of the deltid tuberosity. Quantification of cortical analyses for BMD (mg/cc) and cortical thickness (mm) were performed on each sample using the Advanced Bone Analyses Application in the MicroView software.

3.2.7.3 Trabecular bone analyses

Trabecular bone analyses was performed using microCT images of humeri using MicroView software. The VOI (0.8 mm x 0.9 mm x 0.7 mm) was proximally limited to the appearance of the growth plate in the proximal humeri. The VOI was positioned in the middle of the metaphysis, in order to maximize trabecular bone, while excluding
cortical bone from the analyses. Quantification of trabecular analyses for BV/TV (%),
connectivity (1/mm$^3$) and trabecular spacing (mm) were performed using the Advanced
Bone Analyses Application in the MicroView software.

3.2.8 Statistical analysis
All data were collected from at least 3 independent pairs of littermates. Data was
expressed as mean ±SEM, and p values under 0.05 were considered significant. For
general measurements, statistical significance was determined by t-test analysis
comparing control to KO littermates using GraphPad Prism 3.00 for Windows. Western
blot densitometry data were normalized to controls and compared by t-test. Comparison
of multiple measurements was performed using one-way Anova and a Tukey post test.

3.3 Results
3.3.1 Col1a1-driven Gsk3b inactivation
To study the roles of Gsk3b in skeletal development, we crossed mice with floxed alleles
of GSK-3β (Gsk3b$^{fl/fl}$)(17,22-24) with mice expressing cre recombinase under control of
the 3.6kb fragment of the rat Col1a1 promoter (25,26). The following nomenclature is
used throughout this paper for the genotype of the mice: Gsk3b$^{+/+}$cre$^{-}$ and Gsk3b$^{fl/fl}$ cre$^{−}$ -
referred to as control (Cont), Gsk3b$^{fl/+}$cre$^{+}$ - referred to as heterozygote (Het), and
Gsk3b$^{fl/fl}$ cre$^{+}$ - referred to as knockout (KO). Recombination was detected in DNA
extracted from Het and KO mice using PCR (Fig. 3.1A).

Western blot analyses revealed that cre protein was expressed at high levels in KO
calvarial tissue (bone) and to a lesser extent in growth plate cartilage of P0 KO mice (Fig.
3.2A). Cre protein was not detectable by Western blot in other tissues of KO mice,
Figure 3.1 (17) - Effective recombination

PCR with GSK-3β (Top) and cre (bottom) primers. GSK-3β primers show floxed (fl), wildtype (wt) and recombinant (rec) alleles. The cre primers indicate which mice are cre positive.
including heart (Fig. 3.2A), brain, skin and liver (data not shown). To further examine the skeletal tissue specificity of cre expression, immunohistochemistry (IHC) was performed on P0 tibia sections (Fig. 3.2A). As expected, cre protein was detected in the perichondrium and in cells surrounding the trabeculae. Slight staining for cre was also seen by IHC in prehypertrophic/hypertrophic cartilage. Next, GSK-3β protein expression was examined to determine if Cre expression corresponds to loss of GSK-3β protein (Fig. 3.2B). Quantitative densitometry of Western blots demonstrated that calvarial GSK-3β protein levels were reduced by 70%. This loss of GSK-3β in bone cells was confirmed by IHC (Fig. 3.2B). GSK-3β protein was also reduced in cartilage, to 55% of control, specifically in late hypertrophic chondrocytes as shown by IHC (Fig. 3.2B).

To determine if the ablation of GSK-3β protein would affect the phosphorylation and therefore activity of remaining GSK-3β, the levels of phosphorylation was examined (Fig. 3.2C). The amount of phosphorylated (inactive) GSK-3β was reduced in both KO calvariae (to 43% of control) and cartilage (to 62% of control) samples. Coll-GSK3β−/− mice showed an increase in GSK-3α protein expression in cartilage, whereas GSK-3α was almost undetectable in bone lysates compared to GSK-3β and cartilage samples (Fig. 3.2D). Elevated GSK-3α in mutant cartilage was confirmed by IHC analyses. These results suggest that GSK-3β ablation causes a tissue- and differentiation stage-specific upregulation of GSK-3α protein in growth plate cartilage, in agreement with our earlier study (22), but not in bone tissue.
**Figure 3.2 (18) – Bone-specific inactivation of the Gsk3b gene**

(A) Analyses of cre protein expression in skeletal tissue. **Top:** Western blot analyses of newborn (P0) protein extracts with cre antibody (loading control β-actin) for control (Gsk3b/fl/fl cre− or Gsk3b/fl/+ cre−, C) and KO (Gsk3b/fl/fl cre+; K) mice, using heart (Hr), cartilage (Cart) and calvariae (Calv) lysates. **Bottom:** IHC of P0 tibiae shows that cre protein is present in perichondrium (P), in trabecular (T), prehypertrophic chondrocytes (PH) and cortical bone (C).

(B) GSK-3β protein expression in cartilage and bone tissue. **Top:** Western blot analyses of P0 samples with GSK-3β antibodies and quantitative densitometry results below of cartilage (n=8) (**left**) and calvaria (n=8) (**right**) samples. **Bottom:** IHC of P0 tibia sections probed with GSK-3β antibody.

(C) Analyses of phosphorylation levels of GSK-3β protein. **Top:** Western blot analysis and **Bottom:** Densitometric quantification of the amount of pGSK-3β in control (C or Cont) or bone specific KO (K or KO) mice, in cartilage (n=4) (**left**) and calvaria (n=4) (**right**).

(D) Analyses of GSK-3α protein levels in skeleton of bone-specific GSK-3β KO mice. **Left:** Densitometry of GSK-3α protein expression from Western blot analyses of tissue samples of cartilage (n=8) and calvariae (n=8) from control (Cont) and knockout (KO) mice (loading control β-actin). **Right:** IHC conducted on P0 tibia section using GSK-3α antibodies, with the pre-hypertrophic/hypertrophic growth plate zone displayed.

Quantitative data were compared using t-test analysis, significant differences are denoted by asterisk (*: p < 0.05).
3.3.2 Decreased weight, delayed postnatal bone growth, and altered growth plate morphology in mutant mice

The number of KO mice born was slightly higher than the expected Mendelian ratio (47 KO mice out of 154 total mice), demonstrating that KO mice are viable at birth. Of the 154 pups born, 17 died on the day of birth (P0), and 13 of these were KO mice. Of the 4 control mice that died, 3 were victims of parental infanticide and the fourth died for reasons unknown. The KO mice that died were born alive and died within the first 12 hours. There was no obvious abnormality associated with the deaths; the pups fed and did not appear to suffer from respiratory distress.

Surviving KO mice were significantly (11%) lighter than their control littermates at P0 (Fig. 3.3A). This difference in weight increased to about 26% by P10 and remained relatively consistent (27%) by P21 (Fig. 3.3A). There was no significant difference in weight between heterozygous and control mice (Fig. 3.3A). The greatest effect on KO animal weight occurred between birth and P10, and there was no sex-specific difference in the weight amongst the genotypes (data not shown).

We next examined the length of endochondral bones. There was no significant difference between control and KO tibia length at P0; however, by P21 KO tibiae were approximately 15% shorter (Fig. 3.3B). Thus, the decrease in animal weight preceded the reduction in bone growth and was much greater than the decrease in skeletal length (27% vs. 15%). We evaluated serum levels of insulin-like growth factor 1 (IGF1) and growth hormone (GH) in weaned mice to determine whether reduced growth was associated with changes in these hormones. However, both IGF1 and GH were unchanged in the skeletal-specific KO mice (Fig. 3.4).
The observed reduction of GSK-3β protein in growth plate cartilage in mutant mice (Fig. 3.2B) along with the decreased bone length (Fig. 3.3B) prompted us to examine the organization of the growth plate in KO mice. The growth plates of P0 KO mice showed similar overall organization relative to control growth plates (Fig. 3.3C). Upon closer inspection of the growth plate zones, subtle phenotypes emerged (Fig. 3.3C). The cells of the resting zone appeared larger with more cytoplasmic area in KO animals (Fig. 3.3C r), potentially indicating earlier onset of chondrocyte hypertrophy in areas of future secondary ossification. The proliferating zone of KO mice appeared to be hypercellular, possibly indicating increased proliferation, and the cells appeared flatter (Fig. 3.3C p). The growth plate length from the articular surface to the trabecular bone was not significantly different between P0 KO and control mice. However, when the individual zones of the growth plate were measured, a significant increase in the length of the hypertrophic zone of KO mice was discovered (Fig. 3.3C h, graph). There were no significant differences in the length of proliferating and resting zones. Similar phenotypes were seen in the femur (data not shown).

To determine whether the growth plate phenotype seen in the P0 mice would persist, we examined P21 growth plates (Fig. 3.3D). There was a significant increase in total growth plate length in KO mice (Fig. 3.3D): however, the hypertrophic zone in the KO animals was no longer significantly longer than that of control littermates.

3.3.3 Delayed skeletal development and ossification in KO mice

Skeletal malformations (e.g. of palate and sternum) have been previously observed in global GSK-3β knockout mice (20). We examined our KO model for a similar skeletal phenotype. P0 skeletons were stained with Alizarin Red (bone) and Alcian Blue
Figure 3.3 (19) – Reduced weight and growth plate changes on KO mice

(A) Comparison of postnatal mouse body weight. Weights of control (Cont), heterozygous (Het) and knockout mice (KO) were compared at P0 (n=8) (left), P10, (n=13) (middle) and P21(n=11) (right).

(B) Lengths of tibiae were compared at P0 (n=13) (top) and P21 (n=4) (bottom).

(C) (Top) Safranin O Stain staining of sections, with cartilage glycosaminoglycans stained red. (GP – growth plate; r – resting zone; p – proliferating zone; h – hypertrophic zone) (bottom) Higher magnification of growth plate zones, with the same orientation as in A (articular surface [art] at top and trabecular bone [trab] at bottom). (bottom right) Length of hypertrophic (h) zone at P0. Statistical analysis was conducted using paired (littermates) t-test (n=6).

(D) (Left) Comparison using Von Kossa/Toluidine blue staining of P21 tibia growth plates, cartilage stained in purple and mineralized tissue in black. The orientation is displayed on right of control figure, with secondary ossification center (2nd oss) on top and trabecular bone (trab) at bottom. (right) Length of P21 growth plate. Statistical analysis was conducted using a paired t-test (sex matched littermates) (n=3).

Quantitative data were compared using t-test analysis, significant differences are denoted by asterisk (*: p < 0.05).
Figure 3.4 (20) – Serum IGF and GH

IGF and GH serum levels for weaned mice.
(cartilage) (Fig. 3.5, Fig. 3.6A,B). Our KO mice demonstrated delayed hard palate closure and bifid sternum (Fig. 3.5A), although these defects were not as severe as those described previously in the global GSK-3β KO mice (20). There were no observable differences in the palate of KO mice compared to controls at P21, suggesting a developmental delay rather than a block in development (data not shown). The delay in hard palate closure did not affect the soft palate and mice appeared to feed normally. At P21 the KO sternum was no longer bifid; however, the mineralization of the Xiphoid process seen in the control mice was absent in the KO littermate (Fig. 3.5A, circled).

Several previously undescribed skeletal phenotypes were also evident at P21. Limbs appeared morphologically normal (but slightly shorter) with the exception of the deltoid tuberosity that was clearly underdeveloped and more rounded in KO mice (Fig. 3.5B). The deltoid tuberosity forms through endochondral bone formation involving interaction between tendon and cartilage (32). Further studies showed that the deltoid tuberosity is already smaller at P0 (Fig. 3.5B) and remained underdeveloped even at 1 year of age (data not shown). Postnatal ossified structures of the knee joint such as the patella and sesamoid bones were much smaller in mutant mice (Fig. 3.5C, circled). Delayed KO pelvic bone closure was also observed at P21 that involves the conversion of cartilage to bone (Fig. 3.5D, circled). KO P21 vertebrae were underdeveloped and failed to fuse (Fig. 3.5E, P0 Fig. 3.6A). The most obvious defect was the absence of the large spinous process of the T2 vertebrae (Fig. 3.5D left). Other apparent closure failures were observed in the KO vertebrae associated with the false and floating ribs, as shown for T10 and T13 (Fig. 3.5D). Most other vertebrae appeared normal and closed but had underdeveloped spinous processes (data not shown). Thus, KO mice displayed numerous
Figure 3.5 (21) – Delayed skeletal development and ossification on KO mice

Panels A-E show Alizarin Red (Bone) and Alcian Blue (Cartilage) staining of skeletal elements, comparing Control (Cont) and skeleton-specific GSK-3β knockout mice (KO):

(A) P0 hard palate (top left) P0 Sternum (Top right), (Bottom) P21 circled Xyphoid process

(B) Deltoid tuberosity, (Top) P0, (Bottom) P21

(C) knee joint, (Top) P0, (Bottom) P21

(D) pelvic bone, (Top) P0, (Bottom) P21

(E) T2 vertebrae (left), T10 vertebrae (middle), T13 vertebrae (right).
Figure 3.6 (22) – P0 delayed skeletal development

(A) P0 vertebrae demonstrating wider gap between ossified elements

(B) P0 skulls illustrating larger space between the lambdoid and sagittal sutures
examples of delayed development and ossification of postnatal skeletal elements formed through endochondral ossification.

3.3.4 Suture closure and strength as well as snout length are altered in KO mice

We next inquired whether intramembranous bones were also affected by deletion of osteoblast GSK-3β. Calvarial bones of the skull are formed through intramembranous ossification and defects are often seen as suture malformations where the flat bones meet. Measurements of the skull and calvaria/suture length (diagrammed Fig. 3.7A, left) indicated that the skulls of KO mice were 10% shorter than controls at P21 (Fig. 3.7A). However, the sagittal and frontal sutures were not significantly shorter, indicating that a shorter snout (nasal suture; 17% shorter) was the main reason for the decreased skull length in the KO mice (Fig. 3.7A).

At P21 a delay in cranial suture closure was observed; the occipital and lambdoid sutures are displayed in Fig. 3.7B as examples, but similar phenotypes were observed for sagittal and frontal sutures at P0 (Fig. 3.6B). The strength of the sutures was noticeably reduced in KO mice since during tissue processing (when solution is changed from low to high osmolarity) the suture failed (opened) at a much higher frequency than occurred for control littermates. Long-term studies confirmed weaker sutures in the KO mice as even at one year of age (where no sutures from control mice failed during processing), KO sutures still separated frequently (Fig. 3.7C). The pattern of the sutures was also noticeably altered in KO mice which displayed less interdigitated sutures, an indicator of reduced suture strength (33-36), as displayed in the lambdoid suture tracing in Fig. 3.7C.
P21 KO mice also showed an altered suture pattern in the anterior frontal suture (Fig. 3.7D) as it was split into two with bone in the middle whereas control mice display the
Figure 3.7 (23) – Delayed suture closure in KO mice

(A) Examination of calvarial sutures using Alizarin Red (Bone) and Alcian Blue (Cartilage) staining. **Right:** Length measurements of the skull as shown by the red arrows. **Right Middle:** Assessment of longitudinal length (nose to back of skull) of skull sutures along the midline (n=4). **Left Middle:** Sutures length relative to control (n=4). **Left:** Representative picture of the difference in the length of the nasal suture (or snout length) between control and KO mice.

(B) Lateral view of a mouse skull comparing control (Cont) and KO mice showing occipital (O) and lambdoid (L) sutures at P21.

(C) Analysis of sutures at 1 year of age. **Right:** Representative picture of suture failure in 1 year old KO mice, displaying sagittal (S) and lambdoid (L) sutures. **Left:** Analysis of suture formation, showing lambdoid (L) suture near the sagittal (S) suture. Trace of lambdoid suture is shown below the picture.

(D) Comparison of anterior frontal (AF) sutures of control and KO mice at P21. Higher magnification is shown on the right with arrows indicating suture.

(E) Analysis of anterior frontal suture from 1 year old mice. Trace of suture is displayed on the right.

Quantitative data were compared using one-way ANOVA with a Bonferroni’s post-test or by t-test analysis, significant differences are denoted by asterisk (*: p < 0.05).
typical single suture. This phenotype was still present in the majority of the one-year-old KO mice examined (Fig. 3.7E).

Most of P21 phenotypes described are present at P0 (Fig. 3.5), but less dramatic (Fig. 3.6) or have not ossified at P0. By 1 year of age many of the delayed phenotypes have “caught up” in terms of morphology, with the exception of the deltoid tuberosity that remained rounded. Vertebrae appear fused and the T2 spinous process is present at that age, but still smaller (data not shown).

3.3.5 Transiently increased bone formation in KO mice
We next examined the effects of Gsk3b deletion on trabecular bone formation. Collagen production and bone formation/calcification was examined by Picrosirius red and Von Kossa/Toluidine Blue staining of tibiae at P0 (Fig. 3.8A) and P21 (Fig. 3.8B). Picrosirius red staining showed that the length of trabeculae was increased by almost 100% in KO P0 mice (Fig. 3.8A). This data was confirmed with von Kossa-stained sections (data not shown). The mid diaphysis of KO mice also displayed increased mineralized trabecular bone (Fig. 3.8A, circled and transverse section). At P21, the increase in length of the trabeculae was still significant although less dramatic (25%; Fig. 3.8B), but the mid diaphysis trabecular phenotype was no longer apparent (data not shown). In agreement with these data, microcomputed tomography (µCT) analyses of humeri revealed that at P21 there were no significant changes in bone quality (Fig 3.8C), e.g., cortical thickness and BMD (Fig. 3.9A), and trabecular bone volume/total volume, connectivity, and spacing (Fig 3.9B). However, µCT analyses confirmed delayed development of the deltoid tuberosity in mutant animals.
Figure 3.8 (24) – Increased bone formation in KO mice

(A) Analyses of bone formation in P0 bone-specific GSK-3β KO mice. **Top left:** P0 tibia stained with Picrosirius red for fibrillar collagen. **Bottom left:** Length of trabeculae (Black arrows) (n=7). Increased mid diaphysis trabecular bone was observed in KO mice, circled. **Top right:** VonKossa/Toluidine blue stain showing increased mid diaphysis calcification. **Bottom right:** Comparison of P0 tibia transverse sections stained with Von Kossa/Toluidine blue in the tibia mid diaphysis.

(B) P21 tibia sections stained with Von Kossa/Toluidine blue showing longer trabeculae in KO mice, marked with arrows (**top**) and quantified (n=4) (**bottom**).

(C) µCT analyses of P21 humeri. The deltoid tuberosity (DT) and growth plate (GP) are indicated with arrows.
Figure 3.9 (25) – Bone quality characterization

Microcomputed tomography (μCT) of P21 Coll-GSK-3β KO and control (Cont) mice humeri

(A) Cortical bone characterization, thickness and BMD (n=4).

(B) Trabecular bone characterization, bone volume fraction (BV/TV), connectivity, spacing (n=4).
Figure 3.10 (26) – ColII-GSK-3β KO trabecular bone analysis

(A) Analyses of bone formation in P0 cartilage-specific GSK-3β KO mice. **Top left:** P0 tibia stained with Picrosirius red for fibrillar collagen. **Bottom Middle:** Length of trabeculae (Black arrows) (n=5). **Top right:** Von Kossa/Toluidine blue stain showing decreased calcification

(B) μCT analyses of P21 humeri. The deltoid tuberosity (DT) and growth plate (GP) are indicated with arrows.
In comparison, we examined development of trabecular bone in cartilage-specific CollII-GSK-3β KO mice (22). Surprisingly, we observed a decrease in the amount of trabecular bone in the P0 CollII-GSK-3β KO mice, opposite to the phenotype obtained with the Coll-cre driver line (Fig. 3.10A). μCT was conducted on P21 CollII-GSK-3β KO mice and significant differences relative to control mice were not observed (Fig. 3.10B).

The P0 Coll-GSK-3β KO mice show increased trabecular bone but that phenotype was not maintained throughout the post-natal period to P21. The loss of this phenotype could be due to increased bone resorption, similar to heterozygous global KO mice (21). To examine osteoclast activity in our mutant mice, we performed TRAP staining. TRAP staining increased in Coll-, but not in CollII-GSK-3β KO mice (Fig. 3.11). This effect was most apparent at P0 and less at P21, similar to the lengths of trabeculae. We also saw a transient increase in serum Rankl levels at P0 that by weaning changed to a decrease, whereas the serum OPG levels appeared unchanged at both pre- and post- weaning ages (Fig. 3.12A). Interestingly, Western blotting showed that Rankl protein expression was increased significantly in the cartilage of P0 mutant mice but not in the calvarial samples (Fig. 3.12B).

3.3.6 GSK-3β regulates proliferation, cell cycle and apoptosis in skeletal cells

Having demonstrated that loss of GSK-3β in skeletal cells leads to expanded growth plates and longer trabeculae, we next investigated the cellular and molecular mechanisms involved. Expression of proliferating cell nuclear antigen (PCNA), a marker of proliferation, was examined by IHC of P0 tibiae and by Western blot using extracts of cartilage and calvariae (Fig. 3.13A). Both IHC and Western blot showed a significant
Figure 3.11 (27) – Increased osteoclast number in KO mice

(A) Tartrate-resistant Acidic Phosphatase (TRAP) staining for osteoclasts (purple) of tibia of both ColI-GSK-3β (left) and ColII-GSK-3β (right) KO (KO) and Control (Cont) P0 mice (top), and P21 mice (bottom).
Figure 3.12 (28) – RANKL and OPG serum and tissue expression

(A) Serum levels of RANKL were determined by multiplex analysis. Left: Comparison of mice at P0 by t-test reveals no significant difference between genotypes due to variability (n=3). Middle: Serum RANKL levels in weaned mice at 2-6 months (n=8). Right: Serum OPG levels in weaned mice (n=10).

(B) Tissue levels of RANKL in both cartilage (n=5) and calvarial tissue (n=5).

Quantitative data were compared using t-test analysis, significant differences are denoted by asterisk (*: p < 0.05).
increase in PNCA expression in both cartilage and bone of ColI-GSK-3β KO mice. IHC showed that proliferation was increased in the resting and proliferating zones of the growth plate as well as in cells surrounding the trabecular bone (Fig. 3.13A arrows).

In order for differentiating chondrocytes to progress from proliferating to hypertrophic cells, they must exit the cell cycle. The cyclin-dependent kinase inhibitor p57 is a key regulator of cell cycle exit in cartilage (37-39). Western blot analyses demonstrated a 40% increase in p57 in ColI-GSK-3β mutant cartilage that was confirmed by IHC where p57 was seen increased in the prehypertrophic region of the growth plate (Fig. 3.13B). This result was also shown in our recent publication using pharmacological inhibitors of GSK-3β on tibia organ cultures (22). In contrast, there was a significant decrease in calvarial p57 levels in KO mice (Fig. 3.13B).

During cartilage replacement by bone, hypertrophic chondrocytes undergo apoptosis, the efficiency of which affects both the size of the hypertrophic zone and bone length. Apoptosis was examined by TdT-mediated biotin 16-dUTP nick-end labelling (TUNEL) on P0 tibia sections, as well as by IHC and Western blot analyses of caspase 3 cleavage (Fig. 3.13C). Western blot analysis demonstrated a decrease in both pro-caspase 3 and cleaved caspase 3 in mutant cartilage. In contrast, no clear effects of GSK-3β deficiency on cleaved caspase 3 were detectable in calvaria while pro-caspase appeared reduced (Fig. 3.13C). IHC of P0 tibia probed with an antibody against caspase 3 showed a similar decrease in hypertrophic KO cartilage (Fig. 3.13C). TUNEL also demonstrated a decrease in apoptosis in late hypertrophic chondrocytes in KO mice (Fig. 3.13C). Therefore, ColI-driven GSK-3β deletion resulted in enhanced growth plate chondrocyte
**Figure 3.13 (29) – Proliferation, cell cycle and apoptosis**

(A) Examination of cell proliferation using proliferating cell nuclear antigen (PCNA) as a marker. Western blot analyses for PCNA (P) (loading control β-actin (A)) from growth plate cartilage and calvarial extracts of control (C) and knockout (K) mice. Representative IHC of P0 tibia growth plates as well as trabecular bone probed with PCNA antibodies, with increased positive staining in KOs marked by arrows.

(B) Western blot analyses with p57 antibodies and densitometry on growth plate cartilage (n=8). IHC demonstrates that p57 staining is specifically increased in the prehypertrophic cells of the growth plate. **(Bottom)** Densitometry of Western blotting of calvarial extracts shows a significant decrease in p57 (n=7) in mutant samples.

(C) Apoptosis analyses using cleaved caspase 3 and TUNEL assay. **(Top)** Western blot analysis showing reduced levels of total and cleaved caspase 3 in mutant cartilage samples. **(bottom left)** IHC of paraffin sections of P0 tibia, positive cells are stained brown (arrow). **(bottom Right)** P0 tibia probed with a TUNEL assay, positive cells are stained brown (arrows).
proliferation and cell cycle exit, decreased apoptosis as well as increased osteoblast proliferation.

3.3.7 Increased expression of β-catenin in mutant skeletons

To determine the mechanisms underlying the described phenotypes, we examined a mediator of Wnt signaling known to control endochondral ossification and to be directly regulated by GSK-3β, namely the transcription factor β-catenin (40-44). Western blot analyses demonstrated an increase in β-catenin protein in both cartilage and calvariae of ColI-GSK-3β mutant mice (Fig. 3.14A). IHC of P0 tibia revealed that the number of cells exhibiting nuclear β-catenin increased in the resting zone close to the articular surface and in the prehypertrophic zone in KO mice (Fig. 3.14A Resting, Hypertrophic). Although the background staining is high and similar in both control and KO IHCs of trabeculae, there are more darkly stained cells surrounding the trabecular bone in KO sections (Fig. 3.14A Trabeculae). These results suggest that skeleton-specific ablation of GSK-3β increases canonical Wnt/β-catenin signaling in both cartilage and bone tissue.

Sox9 (45-47) and Runx2 (21,48) are transcription factors that are essential for chondrocyte and osteoblast differentiation and are regulated directly by GSK-3β or β-catenin. We observed a tissue-specific up-regulation of Sox9 in cartilage as well as a bone-specific increase of Runx2 protein in our KO mice (Fig. 3.14B). A similar trend in terms of Runx2 protein levels was seen in cartilage lysates, but the degree of variability precluded statistical significance. Thus, GSK-3β regulates the expression of key transcription factors for cartilage and bone development, possibly through β-catenin-independent pathways.
Figure 3.14 (30) – Expression of key transcription factors

(A) Analyses of β-catenin protein. **Left:** Western blot analyses with β-catenin (β-cat) antibody (loading control β-actin (A)) from cartilage (cart) and calvarial (Calv) extracts of control (C) and knockout (K) mice. **Right:** Quantitative densitometry; data were normalized to control sample for cartilage (n=8) and calvaria (n=7). Representative IHC of P0 tibia sections probed with β-catenin antibodies. Arrows indicate nuclear β-catenin.

(B) Western blot analyses with Runx2 and Sox9 antibodies (loading control β-actin (A)) from cartilage (Cart) and calvarial (Calv) extracts of control (Cont) and knockout (K) mice. Blots were then analyzed by quantitative densitometry (n=8).

Quantitative data were compared using t-test analysis, significant differences are denoted by asterisk (*: p < 0.05).
3.4 Discussion

This study has examined the effects of Col I cre-driven inactivation of the \textit{Gsk3b} gene. The resultant effects included delayed development of specific skeletal structures as well as altered osteoblast and osteoclast physiology. The primary cause of these phenotypes appears to be loss of GSK-3\(\beta\) in osteoblasts and potentially perichondral cells. While we saw a reduction in GSK-3\(\beta\) protein in growth plate chondrocytes, our previous study of cartilage-specific ablation of GSK-3\(\beta\) (22) showed none of the phenotypes described here, which is further supported by side-by-side comparisons between ColII-GSK-3\(\beta\) (22) and Coll-GSK-3\(\beta\) KO mice in the current study. For example, cartilage-specific loss of GSK-3\(\beta\) causes the opposite trabecular phenotype as Col1-driven inactivation. Thus, it is unlikely that partial loss of GSK-3\(\beta\) in chondrocytes contributes to the described bone phenotypes. Moreover, we did not observe a reduction in GSK-3\(\beta\) levels in a variety of other tissues, indicating the specificity of gene recombination in our mice.

On the other hand, the numerous cartilage growth plate phenotypes obtained using the ColI cre driver line is startling, especially in light of the absence of phenotypes obtained in cartilage-specific \textit{Gsk3b} KO mice in our earlier study(22). Loss of GSK-3\(\beta\) was less pronounced and, according to our immunohistochemistry data, appeared later in chondrocyte differentiation in this study compared to data from our ColII-GSK-3\(\beta\) mice. Thus, it is unlikely that the cartilage phenotypes observed here are due to loss of GSK-3\(\beta\) in chondrocytes alone and several alternative explanations seem feasible. First, it is quite possible that altered signaling from the perichondrium and/or osteoblasts (both of which show Cre expression) affects chondrocyte physiology. For example, it is plausible that loss of apoptosis-inducing factors from osteoblasts results in reduced apoptosis of
hypertrophic chondrocytes and thus the elongation of the hypertrophic zone. A second possibility is that systemic alterations in metabolism and endocrine signaling in our Col1-GSK-3β KO mice (described in the accompanying manuscript) affect growth plate chondrocytes directly or indirectly.

Our mutants displayed examples of both advanced and delayed ossification. Interestingly, advanced ossification was also observed in heterozygote global GSK-3β KO mice (21) while delayed skeletal development was seen in homozygote global GSK-3β KO mice at birth (20). Furthermore, it has been shown in vitro through both genetic manipulation and use of pharmacological inhibitors that disruption of GSK-3β activity increases bone formation by cultured osteoblasts (21). Advanced ossification, as shown for example by longer trabeculae, is likely due to direct changes in osteoblasts, such as increased cell proliferation and elevated expression of Runx2 and β-catenin in the absence of GSK-3β. In contrast, the examples of delayed ossification could be due to secondary effects, such as increased osteoclast activity or metabolic changes (accompanying manuscript). Furthermore our observations within the growth plate, specifically the delay in hypertrophic apoptosis and cartilage to bone turnover, not only explains the decrease in longitudinal bone growth but some of the skeletal developmental delays. This delay in the conversion of cartilage to bone likely explains the pelvic phenotype and possibly also that of the deltoid tuberosity, which forms through endochondral ossification (32). However, one alternative explanation for the later is that altered signals from the tendon such as BMP4 (32) contribute to the mutant phenotype.
Our ColI-GSK-3β KO mice showed increased proliferation of growth plate chondrocytes as well as increased expression of the cell cycle inhibitor p57, indicating that these rapidly proliferating cells were also becoming hypertrophic at a greater rate. In conjunction with the observed decrease in apoptosis, this data can account for the longer hypertrophic zone seen in P0 KO mice. We also observed increases in osteoblast proliferation, collagen production, calcification and trabecular bone formation, indicating that the tissue-specific reduction in GSK-3β promotes endochondral bone formation. However, these data (increased chondrocyte proliferation and hypertrophy, increased bone formation) seem to contradict the observed reduction in long bone length observed in these mice, and indeed GSK-3 inhibition in tibia organ culture enhances longitudinal bone growth (22). It has also been shown that heterozygous deletion of GSK-3β partially rescues growth retardation caused by deletion of cGMP-dependent protein kinase II (cGKII) (49). Therefore, one would expect that homozygous deletion in skeletal tissue would cause increased bone growth, contrary to what we actually observed. However, while bones are slightly shorter in our KO mice, the effects on whole body weight are more pronounced and precede changes in bone length. Based on our accompanying manuscript, we believe that deletion of GSK-3β in bone results in systemic suppression of body growth through metabolic effects, but the direct roles of GSK-3β in skeletal cells partially compensates for these effects, explaining why bone growth is less affected than overall body size.

While some of the effects observed here are likely due to increased β-catenin levels and thus activation of the canonical Wnt pathway, it is unlikely that this pathway is solely responsible for the phenotypes described here. For example, inactivation of GSK-3β
mediates some of the effects of the PI3K-Akt pathway that acts downstream of many growth factors (10,15,50). Akt1 directly inactivates GSK-3β, and Akt1 KO mice display a phenotype opposite to that described here, especially with regard to chondrocyte hypertrophy and osteoclast activity (28,51). These data suggest that increased GSK-3β activity is responsible for some of the phenotypes observed in Akt1 KO mice.

Furthermore, GSK-3β is a major node connecting other signaling pathways, including hedgehog/Gli (52), BMP/Smad (53), and NFAT (54). GSK-3 is also a central factor connecting transcription to protein synthesis, through its regulation of translation (e.g. TSC2 (55), eIF2B (56), S6K(57)) and energy metabolism (e.g. insulin signaling, glycogen synthesis (58)). Clearly, some of these processes could be affected in our KO mice and contribute to the complex phenotypes observed.

While many function of GSK-3 proteins appear to be ubiquitous, there are several examples of tissue specific roles (16,17,59). We observed several tissue-specific effects, including chondrocyte-specific up-regulation of p57, Sox9, caspase 3 and GSK-3α, and up-regulation of Runx2 by osteoblasts. Runx2 is a known target of GSK-3β (21) and β-catenin (60) in osteoblasts, and therefore its upregulation in KO bone is not surprising. Sox9 is thought to act antagonistically to β-catenin in cartilage (45,61) and is not expressed in hypertrophic chondrocytes, where we observed up-regulation of β-catenin. The observed upregulation of Sox9 is therefore likely a secondary effect, potentially due to altered signaling from the perichondrium (52,62).

The deletion of both Gsk3b alleles specifically in skeletal cells in this study provides a novel model to study the role of GSK-3β in postnatal skeletal development. Our data
suggest that GSK-3β not only affects osteoblast biology directly, but also controls skeletal development indirectly through signaling to the growth plate. While the phenotype of these mutant mice is very complex and requires further characterization, our data already provide novel insights into GSK-3 function in the skeleton and possibly whole body metabolism.

3.5 References


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Chapter 4

4  Skeleton-specific deletion of GSK-3β causes insulin sensitivity and male specific mortality due to possible development of type II diabetes

A version of this article was submitted for publication as an original research article: Gillespie JR, Bush JR, Bell GI, Aubrey LA, Ferron M, Kream B, DiMattia G, Patel S, Woodgett JR, Karsenty G, Hess DA, Beier F. Skeleton-specific deletion of GSK-3β causes insulin sensitivity and male specific mortality due to possible development of type II diabetes. Submitted to JBMR, December 2011.
4.1 Introduction

Glycogen synthase kinase 3 beta (GSK-3β) has been known for over thirty years to be regulated by insulin signaling where it functions to regulate the activity of glycogen synthase (1). More recently, the two mammalian forms of GSK-3 (α and β) have been shown to be downstream of a signaling cascade from the insulin receptor through PI3K and the protein kinase AKT (PKB), which results in GSK-3 α and β inhibition through phosphorylation of Serines 21 and 9, respectively (2,3). However, the role of GSK-3 is much more complex as it also negatively regulates many other signaling pathways such as Wnt (4,5), NFAT (6), PTH (7), and BMP (8). A recent review attempted to classify the many substrates (>100) of GSK-3β into functional activities such as endocrine, growth and development, neurobiology, and immunology (9).

The physiological functions of the two forms of GSK-3 has been highly debated in the literature, sometimes with evidence of overlapping roles and other times distinct roles, often in a tissue-specific manner (10-15). In fact, our own work has shown differential expression patterns within the growth plate of long bones as well as specific upregulation of GSK-3α in GSK-3β-depleted chondrocytes in vivo (12). In contrast, we also show in the accompanying manuscript (accompanying manuscript) that GSK-3α is expressed at low levels in osteoblasts and is not induced in response to the loss of GSK-3β in bone/calvarial tissue. Moreover, our skeleton-specific deletion of the Gsk3b gene through the use of the rat Coll1 promoter to drive Cre recombinase expression (ColI-GSK-3β KO mice), resulted in significantly reduced body weight that preceded a decrease in longitudinal bone growth (Gillespie et al., accompanying manuscript). This suggests that
loss of GSK-3β in bone affects whole body metabolism, a model that is consistent with recent studies demonstrating the role of bone as a regulator of body metabolism (16-18).

Recent publications studying osteoblast-specific insulin receptor knock-out (KO) mice (ObIR-KOs) have demonstrated that osteoblast insulin signaling modulates body metabolism (16,17). These reports determined that insulin signaling influences circulating levels of the hormonal, under-carboxylated form of osteocalcin from osteoblasts through regulation of FoxO1 activity (16) and the Twist2/Runx2 pathway (17). Bone-derived under-carboxylated osteocalcin, in turn, regulates pancreatic beta cell mass and area as well as glucose tolerance and insulin sensitivity. In conjunction with the known role of GSK-3 as an inhibitor of insulin signaling, these data suggest that skeleton-specific inactivation of the Gsk3b gene should cause a metabolic phenotype opposite to that seen for bone-specific inactivation of the insulin receptor (16,17). This prediction was tested in the current study.

4.2 Material and methods

4.2.1 Materials

The following antibodies were utilized in this study: actin A5441, S6 #2317, pS6 #4856, insulin #I2018 (Sigma Chemical Co., St. Louis, MO); goat anti-rabbit hrp sc-2004 (Santa Cruz Biotechnology, Santa Cruz, CA); GSK-3β #9315, pGSK-3β #9336, GSK-3α #9338, (Cell Signaling Technology, Davers, MA). General chemicals and supplies were purchased from Sigma and VWR (Radnor, PA).
4.2.2 Mouse breeding and genotyping

Mice homozygous for floxed Gsk3b alleles (Gsk3b\(^{fl/fl}\)) have been described previously (12,14,19,20)(Gillespie et al, accompanying manuscript) and were crossed with mice expressing cre recombinase under the control of an osteoblast-specific 3.6kb fragment of the rat Coll1a1 promoter (21,22) (accompanying manuscript). Male mice heterozygous for the floxed Gsk3b allele and expressing Coll1a1 cre were backcrossed with homozygous Gsk3b floxed female mice. The offspring from these crosses were analyzed. Mice were housed under a 12 hr light-dark cycle and fed tap water and regular chow ad libitum. All procedures involving animals were approved by the University of Western Ontario Animal Care and Use Committee. PCR genotyping was performed from ear notch DNA using primers 5’-GGGGCAACCTTAATTTCATT-3’ (forward) and 5’-TCTGGGCTATAGCTATCTAGTAACG-3’ (reverse) for GSK-3\(\beta\) for 30 cycles of 96 °C 55 sec, 56.5 °C 45 sec, 68 °C 2:45min to amplify products. The cre transgene was detected using the primers 5’-CACACTGTGTAGTGCTTCGT-3’ (forward) and 5’-CCTCCAAACCATCCAAGAT-3’ (reverse) using 40 cycles of 95 °C 45sec, 58 °C 30sec, 72 °C 1min.

4.2.3 Histology and immunohistochemistry (IHC)

Freshly dissected mouse tissues (kidney, urogenital tract, pancreas) were fixed in 4% paraformaldehyde overnight before embedding and sectioning at the Robarts Research Institute Molecular Pathology Core Facility (London, ON). 5µm sections were dewaxed in xylene followed by a graded series of alcohol washes, 100% x2, 95% x1, 70% x1. Sections were stained with H&E staining. IHC sections were incubated in 3% H\(_2\)O\(_2\) for 15 min at room temperature, followed by incubation in 10 mM sodium citrate at 95 °C for
30-60 min for antigen retrieval and blocking with 5% goat serum in PBS (23-26). Sections were incubated with primary antibody overnight at 4 °C, washed 3 times in PBS, and secondary antibody was applied according to manufacturers’ recommendations. For detection, DAB substrate was used and counterstained with methyl blue. All images were captured with a Leica DME microscope fitted with a Qimaging Micropublisher 5.0 RTV camera using QCapture Pro 5.1 software.

4.2.4 Western blot analyses

Fresh calvariae and cartilage from long bones were dissected from P0 mice in cold Puck’s solution A (PSA) (12,24,27). Organs (kidney and urogenital tract) were dissected and placed directly in RIPA buffer flash frozen and stored at -20 °C overnight, then homogenized, sonicated and centrifuged. Total protein content was determined, and 25-35 μg total protein was loaded per lane into precast NuPAGE Novex Midi Tris-Acetate Gels and separated using the XCell Surelock Mini-cell (Invitrogen) system. Gels were blotted using XCell II Blot Module (Invitrogen) as per manufacturer’s instructions. Blots were blocked in 5% BSA TBST solution for 1 hour, and then probed with primary antibody overnight at 4 °C followed by appropriate secondary antibody (HRP-conjugated) for one hour at room temperature, and the resultant signal was detected using the ECL detection system (Amersham). Representative blots from at least 3 independent pairs of littermates are shown. Quantitative densitometry analysis was conducted using a ChemiImager 5500 system, subtracting background and normalizing to β-actin loading control signal. Densitometry results were converted relative to control, allowing comparison between blots by t-test analysis.
4.2.5 Blood glucose and serum measurements (Millipore Multiplex), glucose tolerance test and insulin tolerance test

Mouse blood glucose measurements were conducted using a Contour glucose meter. The non-fasted or random-fed readings were measured between 9-10 a.m. The fasted readings were taken from mice that had the food source removed for 6hrs. For the glucose tolerance test (GTT), both control and ColI-GSK-3β KO mice were injected IP with 2.5g/kg body weight of glucose after a 5-6hr fast, and measurements were taken from tail vein blood. For the insulin tolerance test (ITT), control and ColI- and ColII-GSK-3β mice were injected IP with 0.2 U/kg body weight of insulin, and glucose readings were taken from a tail vein. Serum readings were performed using a Millipore Multiplex system on the mouse bone panel 2A (Cat No. MBN2A-41K), Adiponectin-Single plex (Cat No.MADPK-71K-ADPN) and Osteocalcin Single Plex (Cat No. MBN-41K-1OC) in the Screening Lab for Immune Disorders, St Joseph Hospital, London, Ontario, Canada. Serum carboxylation state of osteocalcin was measured as described (16).

4.2.6 Statistical analysis

All data were collected from at least 3 independent pairs of littermates. Data was expressed as mean ±SEM, and p values under 0.05 were considered significant. Statistical significance was determined by t-test or 2-way ANOVA analysis comparing control to KO littermates using GraphPad Prism 3.00 for Windows. Western blot densitometry data were normalized to controls in order to compare between blots by t-test.
4.3 Results

4.3.1 Metabolic changes and male specific mortality in skeleton-specific GSK-3β KO mice

In our accompanying study we examined the skeletal effects of using a 3.6 kb fragment of the rat collagen I promoter to specifically delete GSK-3β from bone-forming osteoblasts (Gillespie et al., accompanying manuscript). These mice were dwarfed with shorter bones than control mice, which was in contrast to our ex vivo studies where we recorded an increase in bone growth upon pharmacological inhibition of GSK-3 (12). The dwarfed phenotype was also surprising since GSK-3 serves as a “brake” on many anabolic pathways such as Wnt and Insulin/IGF (1,2,4,28-30). Interestingly, the delay in skeletal growth occurred postnatally and was preceded by a reduction in weight. Here we show that the reduction in length of skeletal elements was not proportional to the reduction in weight (Fig. 4.1A). Each skeletal element is longer in mutant mice when described relative to the animal’s weight. This result indicates that the reduction in weight is largely not due to loss of skeletal weight and likely due to loss of another tissue, such as fat or muscle. One of the KO animals with the most severe weight reduction is shown with its littermate (Fig. 4.1A). The mutant mouse is almost 60% lighter than the littermate control, while the size of its limbs and skull are affected to a far lesser degree (Fig. 4.1A).

In addition, nearly all of the male Coll-GSK-3β KO mice died between weaning and 7 months of age (Fig. 4.1B) whereas only 1 female Coll-GSK-3β and none of the CollII-GSK-3β KO mice died within this interval (12). This male-specific mortality resembles a recent study of low birth weight animals where male mice expire due to development of
### A

**P21 Skeletal Elements**

![Graph showing skeletal element length/weight comparison between Cont and KO groups.](image)

- **Tibia**: Cont - 17.97g, KO - 6.72g
- **Femur**: Cont - 17.97g, KO - 6.72g
- **Pelvis**: Cont - 17.97g, KO - 6.72g
- **Scapula**: Cont - 17.97g, KO - 6.72g
- **Humeral**: Cont - 17.97g, KO - 6.72g
- **Tail**: Cont - 17.97g, KO - 6.72g

### B

**Survival Proportions**

- **n = 59** (Control)
- **n = 18** (Col I Male)
- **n = 15** (Col I Female)
- **n = 9** (Col II Male)
- **n = 17** (Col II Female)

![Graph showing survival proportions over days.](image)

### C

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Figure 4.1 (31) Metabolic phenotype (Glucose) in Coll-GSK-3β KO mice

(A) (right) Longitudinal measurement of skeletal elements in proportion to the animal’s weight for Coll-GSK-3β KO (KO) mice compared with control littermates (Cont) (n=4). (left) An example of a severely underweight mutant mouse (60%) with limb and skull sizes not proportional to its low weight.

(B) Survival curves (Kaplan-Meier Plot) of both male and female of Coll- and CollII-GSK-3β KO mice tracked for 1 year.

(C) Blood glucose levels (mmol/L) of both Coll- and CollII-GSK-3β KO (KO) at birth (P0). The table also lists glucose readings from weaned mice (2-6 months) in both the fasted (6 hrs) and random fed (Fed) state for both male and female Coll-GSK-3β mice. The last row shows the data for male mice that had a glucose reading performed close to their death (1-2 weeks prior to death).

Quantitative data were compared using t-test analysis, significant differences are denoted by asterisk (*: p < 0.05, **: p<0.01).
diabetes (31,32). In light of this similarity and recent findings describing osteoblasts as endocrine regulators of glucose metabolism (16-18,33,34), we examined blood glucose levels (Fig. 4.1C). Interestingly, the blood glucose levels of the bone-specific KO mice were significantly reduced at birth compared to control littermates, which is in contrast to chondrocyte-specific KO mice (12) that showed no changes in blood glucose levels (Fig. 4.1C). Pronounced hypoglycemia is apparent in skeleton-specific KO pups when glucose readings were taken prior to first feeding (data not shown) and could explain the 30% mortality at birth (Gillespie et al, accompanying manuscript). Weaned female mice, ranging from 2-6 months of age, showed a significant decrease in blood glucose after fasting for 6hrs, whereas the male mice did not show a significant difference. The non-fasted or random fed glucose levels were also examined in weaned mice, and both female and male mice showed a reduction in glucose blood levels. The reduction in non-fasted glucose levels appears greater and more consistent in male mice (individual littermate pairs shown in Fig. 4.2A,B). Several of the male mice, which showed reduced non-fasted glucose levels, became hyperglycemic prior to their premature death (Fig. 4.1C), with a maximum reading of 16.9 mmol/l. Furthermore, glucose was detectable in the urine of most of the male KOs prior to death (data not shown).

4.3.2 Serum insulin and leptin levels in skeleton-specific GSK-3β KO mice

To examine potential causes of reduced blood glucose, we examined the levels of several endocrine regulators of metabolism. Serum insulin is significantly decreased at birth and post-weaning in skeleton-specific GSK-3β KO mice (Fig. 4.3A). Both genders displayed
**Figure 4.2 (32) Individual glucose readings**

(A) Individual glucose readings for male mice in the non-fasted or random fed state relative to control.

(B) Individual glucose readings for female mice in the non-fasted or random fed state relative to control.
Figure 4.3 (33) Serum insulin and leptin are decreased in KO mice

(A) Comparison of serum insulin levels (Millipore Multiplex) between control (Cont) and knockout (KO) mice at P0 (n=3) (Left) and in weaned mice (n=8) (Right).

(B) Analyses of phosphorylation level of S6 in ColI-GSK-3β KO mice. Western blot analyses from tissue samples of cartilage (n=5) and calvarial (n=6) protein extracts from control (Cont) and knockout (KO) mice (normalized to total S6).

(C) Determination of GSK-3β expression in various tissues using densitometry of Western blots: fat, liver, kidney, muscle, and pancreas (n = 4).

(D) Examination of serum leptin levels (Millipore Multiplex) in ColI-GSK-3β KO and control mice at P10 (n=5) and in weaned mice (n=8).

(E) Analyses of serum adiponectin levels (Millipore Multiplex) in ColI-GSK-3β KO and control mice at P10 (n=5) and in weaned mice (n=10).

(F) Histology of gonadal adipocytes shows reduced cell size.

Quantitative data were compared using t-test analysis, significant differences are denoted by asterisk (*: p < 0.05).
Figure 4.4 (34) Individual insulin readings

Individual Insulin reading relative to control.
reduced insulin levels post weaning (individual littermate pairs results displayed Fig. 4.4A); interestingly, the one male KO that did not show this trend was the only one to survive to one year of age. We analyzed the phosphorylation of ribosomal protein S6 protein, a downstream target of the insulin/mTOR/S6K pathway (3,35,36). Cartilage and calvarial extracts from skeleton-specific GSK-3β KO mice displayed reduced levels of phosphorylated S6, in agreement with reduced insulin levels and subsequent reduction in mTOR signaling (Fig. 4.3B).

GSK-3β protein levels were examined in metabolically active tissue to confirm that the observed phenotypes were not partially caused by aberrant cre expression and subsequent non-specific GSK-3β deletion (Fig. 4.3C). Although GSK-3β levels actually appeared to trend toward increased levels in several tissues of the KO mice, statistical analyses did not support significant differences in GSK-3β protein levels between control and KO pancreas, liver, fat, muscle or kidney (Fig 4.3C). The GSK-3β levels in the muscle (data not shown) and pancreas (Fig. 4.5) were also measured by ELISA, and no significant differences between control and mutant mice were found.

Examination of other metabolic hormones revealed that the skeleton-specific GSK-3β KO mice displayed significantly reduced serum leptin levels at P10 and after weaning (Fig. 4.3D). The ColI-GSK-3β KO mice also had lower adiponectin serum levels at P10 but not in weaned mice (Fig. 4.3E). Lower leptin and adiponectin levels are indicators of a metabolic phenotype and possibly reduced body fat content. We examined adipocyte histology, and the mutant mice possessed much smaller adipocytes (a representative image from gonadal fat is displayed in Fig. 4.3F). Given the reduction in leptin,
Figure 4.5 (35) GSK-3β ELISA

GSK-3β ELISA data on female pancreas.
Figure 4.6 (36) Food intake

Food intake, (right) food (g) intake per animal per day, (left) food intake relative to animal weight
adiponectin and adipocyte size we investigated whether the mutant mice consumed different amounts of food (Fig. 4.6). Although there was a trend toward less food consumption per day in mutant mice, this was not statistically significant (Fig. 4.6 left). Furthermore, when food consumption was standardized to animal weight, the mutant mice ingested the same amount of food as control mice (Fig. 4.6 right).

We then examined osteocalcin (OCN) since its under-carboxylated form has been identified as an osteoblast-derived hormone that is regulated by the insulin pathway (16-18). However, in weaned, 2-6 month-old, mice we could not detect changes in total or carboxylated osteocalcin (data not shown).

4.3.3 Coll-GSK-3β KO mice display increased insulin sensitivity
Coll-GSK-3β KO mice displayed a phenotype in terms of blood glucose, Runx2 and bone formation (Gillespie et al., accompanying manuscript) that contrasted the bone-specific insulin receptor KO mice (16,17). However, insulin levels were decreased in both models (16,17). Since the presence of both low glucose and low insulin levels in our mice was perplexing, we next examined insulin tolerance/sensitivity. These experiments were conducted in female mice because male mice were dying and changing metabolically from low to high blood glucose at apparently random ages post weaning. However, prior to this change in glucose concentration, the metabolic profile of both genders appeared similar (Fig. 4.1). Insulin tolerance tests (ITT) on female Coll-GSK-3β KO mice revealed that they were significantly more insulin sensitive than their control littermates (Fig. 4.7A). The area under the curve was significantly lower in mutant mice (e.g., mutant mice showed an overall stronger reduction in blood sugar levels in response
to insulin injection; Fig. 4.7A). In contrast, female cartilage-specific GSK-3β KO mice showed similar insulin responses to their control littermates (data not shown).

Moreover, a glucose tolerance test (GTT) showed no difference between KO and control mice (Fig. 4.7A). Finally, histological analysis of the pancreas showed similar insulin immunostaining and islet size between control and Coll-GSK-3β mice (Fig. 4.7B, Fig. 4.8A & B). Both islet number and circumference showed no significant changes in mutant mice (Fig. 4.8A & B). IHC demonstrated no differences in GSK-3β expression in control or mutant mice (Fig. 4.8C), further validating the specificity of our Cre line. Given the Western blot (Fig. 4.3C), IHC (Fig. 4.8C), and ELISA (Fig. 4.5) results for GSK-3β protein levels in our mutant mice, we are confident that GSK-3β was not ablated in pancreatic tissue.

4.3.4 Large bladder at time of death in male Coll-GSK-3β KO mice

We next sought to understand the basis for the consistent premature death of male KO mice. Prior to their death (3-6 hours), we noticed that these mice presented with a hunched posture and a hard mass in their abdomen (Fig. 4.9). The mass was revealed to be a large, distended and full bladder (Fig. 4.9A). The volume of the bladder was almost 5 times that of control littermates and the urine had a reddish colour, suggesting blood was present (Fig. 4.9A). Histology demonstrated that the bladder structure in terms of muscle layers and shape was completely disrupted in the KO male mice that died prematurely (Fig. 4.9B). However, bladders of male mice not obviously unhealthy or disabled, as well as of female mice, were not significantly different from controls (Fig. 4.9B). To ensure that aberrant loss of GSK-3β in the bladder was not responsible for
Figure 4.7 (37) Coll-GSK-3β KO mice show increased insulin sensitivity

(A) (Top) Insulin tolerance test (ITT) for female Coll-GSK-3β KO (KO) compared to control littermates. (Bottom Left) Area under the curve (AUC) analysis for the ITT of the Coll-GSK-3β KO and control mice. (Bottom Right) Glucose tolerance test results for Coll-GSK-3β mice.

(B) Examination of insulin immunohistochemistry and islet size in Coll-GSK-3β mice, Control (Cont) versus KO.

Quantitative data were compared using 2-way Anova or t-test analysis, significant differences are denoted by asterisk (*: p < 0.05).
Figure 4.8 (38) Pancreas is unchanged in mutant mice

(A) Pancreatic islet number for female mice.

(B) Pancreatic islet circumference for female mice.

(C) Immunohistochemistry using GSK-3β antibodies of female pancreas.
Figure 4.9 (39) Enlarged bladders in male mutant mice that expire prematurely

(A) Comparison of bladder size from male Coll-GSK-3β KO (KO) mice that died relative to age-matched control (cont) littermates. (Top Middle) Urine extracted from bladders of KO mice by syringe. (Right) Comparison of bladder volume (n=4).

(B) Histological analysis of the bladder from a male KO mouse that died with an extended bladder (top right panel) relative to age-matched control bladder on the left. The panels below show bladder morphology in male and female mice at 3 months of ages.

(C) Immunohistochemical staining of GSK-3β of male bladders from control and KO mice.

Quantitative data were compared using t-test analysis, significant differences are denoted by asterisk (*: p < 0.05).
this phenotype, IHC was performed on tissue sections (Fig. 4.9C). The cells of the epithelial layer uniformly stained for cytoplasmic GSK-3β in both control and KO mice, whereas the lamina propria was stained more sporadically with increased nuclear reactivity (Fig. 4.9B).

4.3.5 Damage to the corpus cavernosum in male ColI-GSK-3β KO mice

Since the large bladder could be explained by an obstruction in the urinary tract, we dissected and histologically examined the male urogenital tract from the external penis to the pelvic ureter. Male KO mice that died prematurely displayed a large swollen corpus cavernosum (Fig. 4.10). The highest magnification revealed the corpus cavernosum to be filled with red blood cells. The corpus cavernosum is associated with erectile dysfunction and has been studied in obesity and type II diabetes in various mouse models (37-39). Our observations indicated that the higher serum glucose readings in the KO mice predicted a significantly damaged corpus cavernosum with blood present (Fig. 4.11). Interestingly, this phenotype was present in KO mice that were sacrificed even before displaying an extremely distended bladder. GSK-3β IHC showed that both the control and KO bladders have similar levels of staining in the layer of cells surrounding the cavity of the corpus cavernosum (Fig. 4.12A).
Figure 4.10 (40) Male mutant mice display damage to the Corpus Cavernosum

(A) Examination of male urogenital tract of a male Coll-GSK-3β KO (KO) mouse that expired prematurely compared to control littermate (Cont). (Top) Whole-mount display of the urogenital tract showing external penis (Ex), seminiferous vesicles (SV), testis (T), and swollen and dark coloured corpus cavernosum (CC)(circled). (Middle) Histological sections through the urogenital tract showing a blood filled corpus cavernosum in mutant mice (circled), with high magnification images below showing the presence of red blood cells (Bottom).
Figure 4.11 (41) Examples of damage

(Top) Corpus cavernosum of the urogenital tract of male Coll-GSK-3β KO mice that had slightly elevated (Left) and high blood glucose (Right) when compared to their control littermates. (Middle) Higher magnification of the above corpus cavernosum to examine blood cells present. (Bottom) Kidney of male Coll-GSK-3β KO mice that had slightly elevated (Left) and high blood glucose (Right) when compared to their control littermates.
Figure 4.12 (42) GSK-3β Immunohistochemistry of kidney and urogenital tract.

(A) Corpus cavernosum stained (brown) by immunohistochemistry using GSK-3β antibodies.

(B) Immunohistochemistry staining (brown) of GSK-3β protein in renal cortex and higher magnification of tubules and glomeruli.
4.3.6 Kidney damage and hydronephrosis in male ColI-GSK-3β KO mice

The pathological changes in the corpus cavernosum prompted us to investigate whether there was damage further upstream in the urogenital tract that could be responsible for this phenotype. The kidney of mutant male mice was clearly abnormal (Figures 4.13 and 4.14). The calyces (funnel like structures where the collecting ducts pool and leave the kidney) of ColI-GSK-3β KO were distended, a hallmark of hydronephrosis (Fig. 4.13). The collecting duct renal pyramids also showed signs of dilation and have lost some of their characteristic “pyramidal” histology (Fig. 4.13). Abnormalities can also be seen in the renal cortex where holes/spaces were seen in the mutant (Fig. 4.14). The tubule lining cells appeared damaged and the luminal space enlarged and full of proteinacious material (Fig. 4.14). Also, the glomerular space was also not as well defined in KO animals (Fig. 4.14). This phenotype, similar to the previously described damage to the corpus cavernosum, appears more pronounced in animals with abnormally high glucose levels (Fig. 4.11). The kidney damage was also present in animals that had yet to display enlarged bladders, indicating that hydronephrosis preceded the bladder phenotype. Furthermore, we examined GSK-3β levels in the kidney to ensure that it was not deleted by aberrant cre expression and found similar IHC staining in the glomerulus and renal tubules of mutant and control mice (Fig. 4.12B), in agreement with our Western blot analyses (Fig. 4.3C).
Figure 4.13 (43) Evidence of kidney damage and hydronephosis in male mutants

(A) Histological sectioning through the kidney of a male ColI-GSK-3β KO (KO) that expired with a severely distended bladder compared to control littermate (Cont). Figure shows various magnifications to display structures of the whole kidney, calyces, collecting ducts, and renal pyramids. Arrows are pointing to the presence of abnormally large spaces in the kidney of the KO male.
Figure 4.14 (44) Renal tubule damage

(A) Histological examination of the renal cortex of from a male Coll-GSK-3β (KO) that died compared to control littermate (Cont). (Middle) Red arrows point to clear spaces indicating lack of tissue integrity and damage in the KO renal cortex near glomeruli. (Bottom) Damage to the renal tubules that are full of proteinaceous material (red arrow). Also the Bowman’s space (BS) is not as defined for the KO glomerulus compared to that in the control mouse glomerulus (black arrow).
4.4 Discussion

Our Coll-driven GSK-3β deletion results in a phenotype that is, in many aspects, opposite to those observed with bone-specific deletion of the insulin receptor gene (ObIR-KO mice) (16,17). This is in agreement with the role of GSK-3 as a negative regulator of IR-induced cellular responses (reviewed in (40)). ObIR-KO mice are larger and weigh more due to increased fat deposits (17), whereas Coll-GSK-3β KO mice weigh less, possess smaller adipocytes and decreased serum leptin levels. ObIR-KO mice also displayed decreased trabecular bone formation (17), while our mice show increased trabecular bone at P0 and P21 (Gillespie et al. accompanying manuscript). The GSK-3β KO mice described herein exhibited reduced blood glucose levels whereas it was increased in ObIR-KO mice (17). We also demonstrated an increase in bone Runx2 expression (Gillespie et al., accompanying manuscript), which was reduced in the ObIR-KOs (17). Our studies indicated an increase in osteoclast numbers (Gillespie et al., accompanying manuscript) whereas IR KO mice displayed decreased bone resorption (16). Lastly, our Coll-GSK-3β KO were initially more insulin sensitive relative to control mice, whereas the ObIR-KO mice are insulin resistant (16,17).

However, one important disconnect is apparent between these mouse models (bone-specific GSK-3β and IR ablation); namely, the reduced serum insulin level seen in both strains. There are several possible explanations for this similarity between the models. At the molecular level, GSK-3 is not the only downstream mediator of IR-PI3K-Akt signaling; thus, loss of GSK-3β is unlikely to completely oppose the effects of loss of the IR. Similarly, GSK-3β is involved in many other signaling pathways, in addition to insulin, again providing a potential explanation why insulin levels do not follow the same
patterns as other parameters in the two mouse lines. However, the most likely explanation is that the observed increase in insulin sensitivity caused a secondary reduction in insulin levels in our GSK-3β KO mice. Increased insulin sensitivity would cause lower blood glucose, which could subsequently result in reduced insulin levels in our GSK-3β KO mice.

Another important difference between our mice and those with osteoblast-specific inactivation of the insulin receptor is that we did not observe changes in circulating total or undercarboxylated osteocalcin. In agreement with these data, no changes in the endocrine pancreas or in glucose tolerance were seen in our skeletal GSK-3β KO mice. The reductions in leptin and adiponectin in our mutant mice are possibly secondary to the primary metabolic phenotype, namely the reduction in blood glucose and decreased size of adipocytes. Consequently, the metabolic differences in our mutant mice are possibly due to a novel molecular connection between bone and body metabolism that acts in parallel to osteocalcin-mediated effects. In particular, that pathway might regulate insulin sensitivity in classical insulin response tissues. By depleting GSK-3β, we activated a part of the insulin signaling cascade in osteoblasts, indicating to the osteoblast that the body had excess energy (glucose). Through a still unknown mechanism these osteoblasts then cause increased insulin sensitivity throughout the body.

Furthermore, the phenotype of adipocytes in the mutant mice suggests that they do not obtain sufficient glucose to maintain fat storage levels. Reducing GSK-3 activity has been shown to increase glucose uptake (41), indicating that increased glucose uptake by mutant osteoblasts could deprive other tissues of this energy source. Such a scenario is in
agreement with our observation that the reduction in bone growth in mutant mice occurs at a later time point during development and is less pronounced than body weight reduction.

The premature death of male KO mice is one of the most intriguing aspects of our study. Recently, low birth weight (as observed in our mutant mice (Gillespie et al., accompanying manuscript)) has been shown to contribute to male mouse-specific development of type II diabetes, leading to premature death (31,32). Indeed, our male mice show signs of type II diabetes including a trend toward increased blood glucose as they age, the presence of glucose in their urine, large bladder size and hydronephosis (42,43). The large bladder phenotype is restricted to dying mutant mice whereas kidney and urogenital damage were present in KO mice that were sacrificed prior to phenotypic death. This suggests that the mice are dying of complications due to kidney failure and that the bladder phenotype is the end stage. Hydronephrosis and nephropathy is observed in mouse (44) and rat (45) models of type II diabetes as well as in human type II diabetes patients (46). Moreover, kidney failure is a major cause of death from type II diabetes in humans (47). Interestingly, we recently discovered that one female ColI-GSK-3β KO died at 1.5 years of age. This mouse displayed the large bladder seen in the males which, suggest that this phenotype is not strictly a male phenotype, but that the males are much more susceptible. Higher susceptibility to development of diabetes in males has been shown in mouse models previously (31,48), and faster progression of renal disease is associated with diabetes in human males (49).

Finally, a recent study described a mutation in patients that lead to both hypoglycemia and lack of plasma insulin (50). These patients were found to have an activating
mutation in the AKT2 gene (50). Since AKT is a direct inhibitor of GSK-3 activity, the mechanisms involved in the human disease and our mutant mice might be related. The fact that we see these phenotypes upon skeleton-specific ablation of GSK-3β further highlights the role of bone as a central regulator of body metabolism.

In conclusion, our study demonstrates a key role for of bone GSK-3β in the control of whole body metabolism. Further investigations into the mechanisms involved and the role of GSK-3β in this context will provide important insights with relevance to both skeletal health and metabolic diseases.

4.5 References


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Chapter 5

5 General Discussion and Conclusion
5.1 Overall Rationale

The focus of this thesis was to investigate the role of GSK-3β in skeletal development. Although the skeletal roles of proteins upstream or downstream of GSK-3β have been analyzed extensively, I felt that GSK-3β was a unique kinase and deserved closer examination. GSK-3β is much more than a regulator of individual pathways, such as the Wnt pathway or the insulin/IGF pathway. Rather, GSK-3β is a node of regulation for many anabolic pathways within a cell. The web of pathways feeding into and leading out of GSK-3β appears to vary depending on the cell type and may even vary depending on context, which explains the high level of discrepancy in the literature. Understanding GSK-3β’s role in a given tissue is extremely important. This importance is highlighted by the physiology and biochemical nature of GSK-3β itself. For one thing, there are at least two completely different ways to inhibit GSK-3β activity, through phosphorylation or disruption of the destruction complex. In various studies these two pathways have been shown to crosstalk, while in others they have been shown to be completely separate (1-3). To further complicate things there is another protein, GSK-3α, that shares almost complete sequence homology, but is encoded but a separate gene. Why would evolution conserve two proteins that were almost identical? The likely answer is the shear importance of GSK-3’s role. Indeed it has been shown in many studies that the two GSK-3 proteins can overlap in function and compensate for each other; however, other studies have revealed completely separate roles and pathways that they regulate (3-6). Historically GSK-3α has been largely ignored and the primary focus has been on GSK-3β, but I believe (partially based on our data) that greater attention needs to be given to GSK-3α.
5.2 Study 1

5.2.1 Experimental Rationale

To explore the role of GSK-3β in skeletal development I employed tissue-specific genetic manipulation \textit{in vivo}. The use of pharmacological GSK-3 inhibitors leads to increased bone mass in both mice and rats (7,8). Genetic manipulation has also shown the role of GSK-3β in skeletal development in several studies. Ubiquitous deletion of GSK-3β causes cleft palates, bifid sternum and delayed ossification of sternum, skull, ear bones, and cranial base (9). Heterozygous deletion of GSK-3β also caused a skeletal phenotype, namely increased ossification, clavicle abnormalities and increased bone reabsorption (10). The sum of these studies identify three things; that GSK-3β appears to be important for skeletal development, that GSK-3α does not compensate completely for the loss of GSK-3β, and that there are opposing phenotypes with one copy of an allele compared to the loss of both. The last point is very interesting and suggests that the role of GSK-3β in bone development is complex. I identified a lack in the literature for GSK-3β and skeletal development. One under examined area was that no one had identified whether or not the skeletal effects seen in the whole body GSK-3β heterozygous and homozygous mutants were cell autonomous to skeletal tissues, or whether they are a secondary effect to other phenotypes (i.e. result of knocking out GSK-3β in the brain). Another gap was that no one was able to observe the effect of deleting both alleles of GSK-3β on postnatal growth and development, since homozygote whole body mutants show perinatal lethality (9,11). Therefore I sought to explore these issues by using three models: 1. \textit{Ex vivo} tibia organ culture treated with pharmacological inhibitors of GSK-3, 2. \textit{In vivo} cartilage-specific GSK-3β deletion, and 3. \textit{In vivo} bone-specific GSK-3β deletion.
5.2.2  Aim 1:

To determine the role of GSK-3β plays in growth plate function, endochondral bone formation and bone growth.

5.2.3  Hypothesis 1:

Deletion or inhibition of GSK-3β in cartilage causes increased endochondral bone growth.

5.2.4  Result Summary 1:

I found that treatment of tibia organ cultures with a GSK-3 inhibitor caused the tibia to grow more over the 6 days in culture. This was expected as I released the anabolic break, GSK-3. Histological analyses showed that the resting/proliferating zone was longer but the hypertrophic zone was shorter upon inhibitor treatment, yielding an overall growth plate length that was not significantly different. Instead, increased length of treated bones was due to an expanded mineralized zone. This data together suggested that increased bone growth was due to increased proliferation, hence the longer resting/proliferating zone, and increased turn over of hypertrophic chondrocytes, hence the shorter hypertrophic zone and longer mineralized area.

Another thing that production of cartilage-specific GSK-3β KO should tell us is whether or not the skeletal defects seen in the whole body KO were due to alterations in the cartilage (9). The cartilage-specific GSK-3β KO was produced with approximately 80%
reduction in GSK-3β protein in cartilage. However, the lengths of the KO bones were not significantly different from the control littermates, and no skeletal phenotypes resembling those of whole body mutant mice were seen. No histological differences were seen in the growth plate of cartilage-specific KO mice, suggesting that the effects observed in our organ cultures were due at least in part to GSK-3α activity. Levels of β-catenin protein in the pre-hypertrophic zone, where GSK-3β is most highly expressed, was increased in both the ex vivo and in vivo studies. In addition, my data show that GSK-3α is upregulated to compensate for the loss of GSK-3β protein.

5.2.5  Conclusion 1:
Endochondral bone growth is regulated by one (or both) of the GSK-3 proteins, as seen in the organ culture experiments, but the in vivo study of the cartilage-specific GSK-3β KO revealed that GSK-3β was not essential for regulating bone growth. When reviewing the literature, I see that the GSK-3α global KO is not different in terms of size (12,13), and the GSK-3β global KO is lethal with skeletal abnormalities (cleft palate and bifurcated sternum) (9). The heterozygous GSK-3β mice were also shown to have increased bone mineral density (10). Taken together this data suggests: 1. That likely both Gsk3 genes have to be deleted or inhibited in chondrocytes to effect growth, 2. That GSK-3β affects bone development but not through regulation of chondrocyte function.
5.2.6 Limitations of experimental design 1:

I decided to use tibia organ culture experiments to examine growth over six days in culture using pharmacological inhibitors of GSK-3. These experiments have several advantages for studying growth plate chondrocytes: 1. The chondrocytes are assembled in fully functioning growth plates with all the zones, 2. The tibiae are in a 3D structure as it would be in vivo, 3. The tibiae grow outward as they would in vivo, 4. The tibiae contain other cell types that are present in in vivo bones such as osteoblasts, osteoclasts, and perichondral cells, 5. They are easy to culture and treat, with longitudinal growth as one measureable outcome. In other words, the tibia organ culture system is as close as possible to in vivo studies but still maintains the functionality of culturing (e.g. exact control of culture medium, ease of pharmacological manipulation etc.). However, there are several important factors that are missing in the organ culture system versus an in vivo system, such as mechanical stress from the movement and weight of the animal as well as the influence of the circulatory system. All things considered the tibia organ culture system is useful to test effects of pharmacological compounds on bone growth.

One thing important thing to clarify is that the GSK-3 inhibitors I used in the organ culture experiments inhibit both α and β forms. This information is important to interpret the data correctly especially when comparing the tibia organ culture day with the in vivo data. Since ex vivo experiments showed that GSK-3 was responsible for regulating endochondral bone growth, I felt a cartilage-specific GSK-3β deletion in vivo study was warranted. At first glance the fact that the pharmacological inhibitors inhibits both forms of GSK-3 appears as a weakness. However, knowing this weakness I can turn it into
strength by helping us interpret the data by concluding that both GSK-3s must be inhibited in order to effect bone growth.

5.3 Study 2

5.3.1 Experimental Rationale:
Previous studies showed that GSK-3β controls bone development (9), but no one determined if this was cell autonomous for skeletal cells, and no one was able to study postnatal development. Study 1 showed that the skeletal effects were not a result of deleting GSK-3β from chondrocytes. For this study I set out to produce osteoblast-specific GSK-3β KO mice to determine its role in skeletal development.

5.3.2 Aim 2:
To determine the role of GSK-3β signaling in osteoblasts during postnatal bone development.

5.3.3 Hypothesis 2:
Deletion of GSK-3β in osteoblast causes increased bone formation.
5.3.4 Result Summary 2:

The first observed phenotype of the bone-specific GSK-3β KO mice was that approximately 30% of the KO mice were dying at birth or shortly after. Some of these mice were never observed alive; others survived for hours and appeared to be normal and healthy. Most of the dead pups contained milk in their stomachs and appeared to feed properly. This was a very interesting phenotype as the global GSK-3β KO mice had 100% mortality at birth (9). This suggests that among all the tissues that GSK-3β was deleted from in the global KO, bone contributed to the mortality.

The KO mice that survived weighed less than their control littermates. This difference was about 13% at birth (P0) and increased to 26% by 10 days of age (P10) after which it remained relatively constant through P21 and beyond. There was no difference between the heterozygous mice and the controls, which indicates that a homozygous deletion of GSK-3β is needed to cause the weight phenotype. This theory is supported in the literature as the heterozygous global KO did not display any size or weight phenotype (10). Since I deleted GSK-3β specifically in bone I investigated whether the weight loss was due to a loss in bone mass. The P0 KO mice had similar lengths of bone to the control mice; however, by P21 the length of the KO long bones were significantly shorter. This was an interesting result for two reasons: 1. P0 mice weighed less but their skeleton was of normal size, and 2. After birth skeletal growth was reduced. Therefore the weight must be lost from another tissue, which was really interesting in our bone-specific mutants. The other surprising result was that bone growth was reduced while I would expect increased bone growth from the results of our tibia organ culture experiments (14). Since growth was affected I examined the growth plate for clues to
why bone growth was delayed. The main effect on the growth plate was a significantly larger hypertrophic zone in the KO mice. Proliferation was increased throughout the proliferating zone, as was the expression of the protein p57 throughout the prehypertrophic zone. These results would suggest that the bone is growing more as more chondrocytes are being produced and more are transitioning or differentiating into hypertrophy. In contrast, apoptosis was reduced in the GSK-3β KO mice which suggests that the chondrocytes were trapped in hypertrophy, preventing the turnover to bone.

I also observed the same increase in GSK-3α in the prehypertrophic zone of the bone-specific KO mice as I had observed in the cartilage-specific GSK-3β KO mice, likely due to leaky cre expression in cartilage. These bone-specific KO mice displayed a phenotype of delayed skeletal development such as a delay in fusing of bony elements in the palate, sternum, pelvis, cranium, and vertebrae. On the other hand, there was also increased bone formed in the P0 mice as shown by longer trabecular bone and higher amounts of bone in the marrow of mid diaphysis. This was likely to the increase in proliferation of osteoblasts. However there was also an increase in the number of osteoclasts that might be responsible for the normalization of the amount of mineralized tissue by P21, where only a small difference could be observed.

I also examined the direct effects of GSK-3β deletion on osteoblast signaling (Figure 5.1). Through the deletion of the negative regulator of both the Wnt and Insulin pathways, GSK-3β, I examined various cellular markers. I observed an increase in protein levels of both β-catenin and Runx2, which are both markers of osteoblast proliferation, differentiation and activity as well as downstream of GSK-3β in the Wnt
pathway (Figure 5.1). Likewise I did observe an increase in osteoblast proliferation determined by PCNA expression and increased osteoblast activity as seen in the increased trabecular bone. Therefore, GSK-3β regulates osteoblast proliferation and activity by controlling levels of β-catenin and Runx2 (Figure 5.1).

5.3.5 Conclusion 2:
From these results it can be concluded that osteoblast GSK-3β regulates skeletal development. Osteoblast GSK-3β is also responsible for the perinatal mortality seen in our KO mice and contributes to the death seen in the global GSK-3β KO study (9). Growth was slower postnatally in KO mice, which was opposite to that seen in the organ culture study (14) and therefore likely caused by secondary effects. P0 KO mice weighed less but skeletal elements were not any smaller, suggesting that bone GSK-3β is also regulating tissue that is not bone, likely through an endocrine manner. This conclusion became much more likely after two papers were published in “Cell” characterizing osteoblast-specific deletion of the insulin receptor (15,16) which acts upstream of GSK-3β.

5.3.6 Limitations of Experimental Design 2:
One major weakness of this study is the nonspecific and leaky expression of the 3.6Kb rat Coll1a1 promoter that was used. The Col I-GSK-3β KO were produced and characterized to have a 70% reduction of GSK-3β in calvaria/bone samples. However, I also found that GSK-3β protein in cartilage was also reduced to 55% of the control. This was
unfortunate as any phenotype discovered could not be attributed with complete certainty to GSK-3β function in osteoblasts. However, given the results of the cartilage-specific KO mice which had no skeletal effects (14) I am confident that any skeletal phenotypes observed are due to the deletion of GSK-3β in osteoblasts.

5.4 Study 3

5.4.1 Experimental Rationale 3:

Our data suggested that weight reduction in our bone-specific mutant mice appeared earlier and was more severe than reduction in skeletal growth. Additionally, in the process of aging the mice I discovered that the male KO mice were dying post weaning between 2-8 months of age. This did not affect the female mice although their phenotypes were similar in terms of weight and skeletal effects. This result suggested that I was indeed dealing with a metabolic phenotype because, how could relative subtle skeletal phenotypes described in chapter 3 cause death and why just male mortality? Two studies looking at the osteoblast-specific insulin-receptor KO mice (15,16) discovered effects on fat content, blood glucose, insulin levels, and insulin sensitivity. The insulin receptor is just upstream of GSK-3β, which suggested that GSK-3β might also affect whole body metabolism. Studies involving low birth weight showed that male mice became more susceptible than females to developing type II diabetes and subsequent death (17-19), prompting us to particularly focus on glucose metabolism in our mutant mice.
5.4.2 Aim 3:
To investigate the whole body metabolic effects of deleting GSK-3β in osteoblasts.

5.4.3 Hypothesis 3:
Deletion of GSK-3β in osteoblasts causes increased insulin signaling, which will in turn affect whole body metabolism.

5.4.4 Result Summary 3:
To explore the hypothesis that osteoblast GSK-3β was regulating whole body metabolism I examined the animals blood glucose levels. The KO animals of both genders had lower blood glucose in the fed state. The KO mice also displayed lower serum insulin levels which was surprising at first glance, but made sense in light of their increased insulin sensitivity since the mutant mice require less insulin to facilitate the uptake of excess blood glucose. I also showed that circulating leptin and adiponectin were lower in our KO mice and that the actual size of the adipocytes was smaller. There was concern that leaky cre expression could be causing these phenotypes, but analyses by IHC, western blotting, and ELISA assays determined that expression of GSK-3β was normal in metabolically active tissue.

To further understand the signaling status within the osteoblasts I tested the phosphorylation status of S6, which is downstream of the insulin receptor but not downstream of GSK-3β (Figure 5.1). The amount of active S6 was decreased in the bone-specific GSK-3β KO mice, indicating that like the lower levels of circulating insulin
Figure 5.1 (45) Signaling pathways within GSK-3β KO osteoblasts

This schematic represents the signaling pathways affected by deletion of GSK-3β in osteoblasts. The molecules that were quantified in the study are boxed in red and their abundance relative to control littermates indicated with either a green arrow for increased or a red arrow for decreased. The cellular effects are indicated outside the cell.
resulted in reduced activity of intracellular mediators. However, insulin-activated pathways downstream of GSK-3β would be activated despite lower levels of insulin. This could be one reason why our model does not show the exact opposite phenotype of the insulin receptor KO models (15,16).

One of the most interesting aspects of bone-specific GSK-3β KO mice is that almost 100% of the male mice die postnatally between 2-8 months of age. Moreover, male mice that displayed lower glucose levels early in their life, changed to high glucose levels prior to premature death. This suggested that these male mice might be more susceptible than females to developing type II diabetes. These male mice developed damage and abnormalities throughout the urogenital track prior to death. The most obvious was an extremely large bladder, 4 times the volume of the control mice, but our analyses showed that damage throughout the track from the corpus cavernosum to the kidneys preceded the bladder phenotype.

5.4.5 Conclusion 3:
Osteoblast GSK-3β regulates whole body metabolism by controlling insulin sensitivity. Male bone-specific GSK-3β KO mice show premature death, possibly because they are more susceptible to developing type II diabetes.

5.4.6 Limitations of Experimental Design 3:
The weakness of this study is similar to second study, namely the nonspecific or leaky expression of cre recombinase. As in study 2 the cartilage-specific KO mice did not display any of the phenotypes described, suggesting that Gsk3b KO in chondrocytes did not contribute to these phenotypes. I also took great care in examining GSK-3β protein
levels in key tissues throughout the body to ensure that GSK-3β was not deleted from other organs of interest. Another weakness is that our studies are not able to pinpoint the cause of metabolic abnormalities and premature death completely.

5.5 Contribution to Current State of Knowledge Regarding GSK-3β and Skeletal Development

My first set of experiments focused on the cartilage growth plate. I first examined the protein expression pattern of both GSK-3α and β throughout the growth plate. This served several purposes: 1. It was important to establish whether both GSK-3 proteins are present in the growth plate, 2. The localization of the kinases could suggest the functions or processes regulated by GSK-3. As mentioned in the introduction, the growth of endochondral bone is determined by the rate in which chondrocytes cycle through the stages of the growth plate. Each stage and the transition between each stage are tightly regulated by various autocrine, paracrine and endocrine signaling pathways. So this simple experiment of localizing the GSK-3 proteins within the growth plate was also one of the most important. I showed that GSK-3α was ubiquitously expressed throughout the growth plate, which suggests a more general role, whereas GSK-3β was primarily found in the pre-hypertrophic/hypertrophic zone, therefore suggesting that GSK-3β may regulate the transition to or maintenance of the hypertrophic cells.

Another important aspect that came out of the organ culture experiments was the relation of GSK-3 to the regulation by PI3K. Through the use of a PI3K inhibitor in double inhibitor experiments I showed that the regulation of the proliferating zone was PI3K-dependent whereas the hypertrophic zone was PI3K-independent. This is an important
contribution to the GSK-3β and cartilage fields as it shows that GSK-3β is regulated by different pathways within the same cell type, suggesting that regulation can change with something as subtle as differentiation stage. This requires greater more specific analysis to determine the mechanism responsible.

One interesting and unexplained result is I still see an increase in β-catenin in the pre-hypertrophic area of the \textit{in vivo} model, suggesting that GSK-3β alone regulates β-catenin in chondrocytes. This fits with the model discussed above where GSK-3 effects on hypertrophy are PI3K-independent and potentially mediated by the canonical Wnt pathway. As to the role that this increased β-catenin is playing in the growth plate I were unable to determine however it appears not to be involved in growth. It has been shown that inhibition of β-catenin in growth plate cartilage causes postnatal runting (20), whereas the over expression of β-catenin in cartilage leads to severe skeletal abnormalities and loss of growth plate organization (21). However, our mice fail to show much of any cartilage phenotype, so it is unknown what the function of the increased β-catenin in the prehypertrophic/hypertrophic zone.

I examined the protein levels of GSK-3α in both cartilage and bone and observed consistent upregulation of GSK-3α protein when GSK-3β is lost from cartilage; however there was no change in the bone tissue. This is a very interesting result; it suggests that this upregulation of GSK-3α is tissue-specific for cartilage, since this effect has not been reported for any other cell type (to our knowledge). Furthermore, in osteoblasts the GSK-3α was not upregulated to compensate for the loss of GSK-3β and GSK-3α was difficult to even detect in the control bone samples. This suggests that in osteoblasts the
primary GSK-3 is the β form, which lent greater credence that deletion of GSK-3β would lead to a phenotype.

Our results on the skeletal phenotype of bone-specific *Gsk3b*<sup>−/−</sup> mice are interesting for a couple of reasons: 1. That these bone-specific KO mice have a cartilage phenotype, and 2. That the phenotype of bone growth is the opposite of the organ culture results. It would appear that the cartilage phenotype is not a direct result from knocking down GSK-3β in the cartilage as the cartilage-specific KO (14) did not display a phenotype and the same suspected compensatory upregulation of GSK-3α was present in both models. Therefore secondary effects are the likely cause of the cartilage phenotype. These secondary effects can occur either through changes in paracrine signaling (e.g. from the osteoblasts or perichondrium) or from metabolic changes throughout the mouse as seen in chapter 4. The fact the effect on growth is the opposite of that of GSK-3 inhibitors in the organ culture model (which contains perichondrium and an ossified core) leads us to believe that the reason for the reduced growth in the ColI-GSK-3β KO mice is metabolic. The fact that weight reduction occurs before the delay in bone growth also lends credence to the metabolic theory.

In our bone-specific KO mice, two different effects appear to act on osteoblasts, the direct removal of the GSK-3β “brake” in osteoblasts themselves as well as the proposed systemic metabolic effect. These two signals result in delayed development and ossification throughout the skeleton, but an increase in trabecular bone. Interestingly, the global heterozygous GSK-3β mouse displayed increased trabecular bone (10) whereas the global homozygous GSK-3β deleted mice displayed skeletal delayed development (9)
whereas I show that both phenotypes are present in my mutant mice at the same time. It appears possible that these seemingly contradictory phenotypes could be due to site-specific effects of GSK-3β deficiency.

Based on the reduced somatic growth of these mutant mice, and in light of recent findings that identify bone as regulator of body metabolism, I examined the alterations in metabolism in our bone-specific Gsk3b KO mice (Figure 5.2). KO mice had lower blood glucose levels, especially in the fed state. This was specific to osteoblast GSK-3β as the cartilage-specific GSK-3β KOs did not show any changes in blood glucose levels (Figure 5.2). At this point two studies were published on osteoblast-specific deletion of the insulin receptor, which effected whole body metabolism, fat content, and insulin sensitivity (15,16). Since GSK-3β is a negative regulator of the insulin pathway, one would expect that our bone-specific GSK-3β KO mice would have the opposite phenotype to the osteoblast-specific insulin receptor KO (Ob-IR KO). Indeed, this is the case for many parameters such as animal weight and blood glucose levels (Figure 5.2).

Since the Ob-IR KO mice had larger fat content, I examined the adipocyte cell size in
Figure 5.2 (46) Effects on whole body metabolic signaling

This schematic depicts the documented and proposed signaling changes throughout the body of the osteoblast-specific GSK-3β KO mice. The parameters boxed in red were measured in the studies. When a parameter was increased compared to the control it is indicated by a green arrow, as decrease by a red arrow and no change by a black equal sign.
the mutant mice and found they were smaller (Figure 5.2). Likewise, circulating levels of two hormones synthesized by adipocytes, leptin and adiponectin (Figure 5.2), were reduced in the bone-specific KO mice. These data suggest that osteoblast GSK-3\(\beta\) regulates whole body metabolism by affecting fat deposition and blood glucose levels.

The role of bone as a regulator of body metabolisms is a relatively new and still controversial concept (15,22,23). Bones are not static; even in adulthood bones are undergoing constant remodeling. This process is responsible for maintaining the balance between the physical demands of the individual to ensure bone integrity, consumption of energy and minerals and weight of the bones. Given the large mass of bone in the body this process requires substantial amount of energy. Thus it makes sense that bone is able to communicate its energy requirements/status to the rest of the body and coordinate metabolic activities with other tissues.

One parameter in my mice that was not opposite to the Ob-IR KO model was serum insulin levels, both mutant strains displayed reduced insulin levels (Figure 5.2). The Ob-IR KO model showed that the reduced insulin was due to a decrease in insulin secretion (15). These mice also displayed a decrease in insulin tolerance, increase in insulin resistance (15,16), which means less blood glucose was taken up by the tissues per specific insulin injection. They also showed as decrease in glucose tolerance (15,16), which means that it took longer for glucose levels to return to normal after a glucose injection. Both of these characteristics explain why the Ob-IR KO mice had increased blood glucose in the fed state, since less insulin is secreted and target tissues are less responsive to insulin to facilitate glucose uptake. These effects were attributed to the reduced production of the bone hormone osteocalcin (Ocn), more specifically the
undercarboxylated form of osteocalcin that directly controls pancreatic islets (15,16). Our Coll-GSK-3β mice were tested for serum Ocn levels, but showed no differences (Figure 5.2). Thus it was not surprising when analysis of the pancreas showed no changes in islet number or size in our mutant mice (Figure 5.2). Nor was there any difference in glucose tolerance of the Coll-GSK-3β mice, which meant that these mice could clear excess glucose from the blood stream as efficient as control mice.

With lower insulin levels in our mutant mice, one would expect higher glucose if all other metabolic parameters are unaffected however, I observed the opposite. There are at least two possibilities to explain this unexpected result: 1. Insulin is more effective at facilitating the uptake of glucose in target tissues; therefore less insulin is needed to uptake excess glucose; or 2. More glucose is used by one or more tissues, resulting in reduced blood glucose, which in turns results in reduced release of insulin. By injecting insulin into fasted mice and observing the effects on blood glucose levels over time, I tested insulin tolerance. Glucose levels were reduced to a larger degree and for an extended period in mutant mice compared to control mice, which suggests that the mutant mice are more insulin sensitive (Figure 5.2). However, disruption of GSK-3β in other tissues has been shown to increase glucose uptake and insulin stimulated glucose uptake (24,25). Although further examination is required, one must wonder, given the size of the skeleton, whether the direct uptake of glucose by osteoblasts could be causing the observed metabolic and skeletal phenotypes? For example if mutant osteoblasts have increased glucose uptake, could this effect be enough to reduce blood glucose and subsequently affect the growth of the rest of the body? The reduction in available glucose could cause the reduction in fat tissue and even growth of the skeleton.
Therefore probably the most important experiment to conduct on these mice is to figure out where the glucose is going, which tissue has the increased insulin sensitivity. I know glucose is not being taken up by the adipocytes, but what about the other classic targets of insulin such as muscle and the liver? If this is the case, then there must be an unknown endocrine signal from osteoblasts to induce glucose uptake in these tissues. The other big question is what about the brain? The brain’s only source of energy is glucose, and brain function is greatly affected by fluctuation in blood glucose levels. Is the brain being starved of glucose as it appears in the rest of the body?

In our Coll-GSK-3β KO mice, I was simulating a constant fed state (by activating the insulin signaling pathway intracellularly) in our osteoblasts, and blood glucose is low (Figure 5.2). In the Ob-IR KO mice they are simulating a fasted state in their osteoblasts and blood glucose is high. When interpreting this data, one has to be extremely cautious as in both cases since I have just altered insulin signaling in bone tissue, whereas the rest of body will be receiving not only natural ebb and flow of insulin and glucose associated with eating but whatever effects the altered osteoblasts have on the rest of the body. There are several aspects of our phenotype that I cannot explain, one being the adipocyte effects. In our Coll-GSK-3β model the KO mice have increased insulin sensitivity yet deceased fat mass and more bone (relative to body weight) (Figure 5.2), whereas the Ob-IR KO mice have the opposite phenotype, more fat, insulin resistance, and less bone. High adiposity has been shown to increase bone mass and vice versa (26), but the opposite is occurring in both our mice and the Ob-IR KO mice.
The final and maybe most interesting aspect of the ColI-GSK-3β KO mice is the sudden death of the male mice. I am unsure if this death is related to death seen in 30% of the KO mice just after birth. Although not tested, I do not believe that these P0 deaths were all male since the ratio of genders for surviving mice was approximately 50:50. This finding is extremely interesting, as I may have found a model for the study of male-specific development of diabetes. Understanding why the females are unaffected is also important and lead to therapeutic treatments of high-risk males.

5.6 Overall Conclusion

I have made some significant contributions to understanding the complex role of GSK-3β in the skeleton and beyond. This greater understanding of GSK-3β function in both cartilage and bone may lead to new insight for researchers studying other tissues. I also successfully showed that these were worthwhile experiments to conduct. Others studies have examined molecules both upstream and downstream of GSK-3β, but none of these models expect the insulin receptor studies (15,16) have shown metabolic changes. Furthermore, although GSK-3β is downstream of the insulin receptor, the phenotype was not the exact opposite, very likely due to GSK-3β’s role in many other signaling pathways. Thus, our studies also contributed to a greater understanding of the complex nature of the regulation of whole body metabolism by bone.
5.7 References:


Appendices

Appendix A: Copyright Permission request Study 1

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Journal: Endocrinology
Author Name: Gillespie JR
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Date: December 8, 2011
Appendix B: Animal use protocol

Western

AUP Number: 2007-045-06
PI Name: Beier, Frank
AUP Title: Regulation Of Endochondral Bone Growth By Hormones

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Regulation Of Endochondral Bone Growth By Hormones"

" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2007-045-06:

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care
Curriculum Vitae

**Education**

2006-Present  **PhD** Candidate (Physiology), University of Western Ontario, London, Ontario

Thesis title: The role of Glycogen Synthase Kinase 3 beta (GSK3\(\beta\)) in endochondral bone development

Supervisor: Dr. Frank Beier

(graduation expected in fall 2012)

2003-2005  **MSc** (Physiology), University of Western Ontario, London, Ontario

Thesis title: Purification, Identification and Characterization of Big Stanniocalcin

Supervisor: Dr. Graham Wagner

2001-2002  **BEd** (Intermediate/Senior Chemistry and Environmental Science), University of Toronto (OISE), Toronto, Ontario

1996-2001  **BSc** (Honours Biochemistry/Biotechnology), University of Waterloo, Waterloo, Ontario

**Research**

**Publications**


Manuscripts in Revision


Manuscripts in Preparation


Abstracts


Gillespie JR, Higgs A, Ulici V, Agoston H, and Beier F. April 2007. GSK-3 Signaling in C-type Natriuretic Peptide Induced Bone Growth. 2nd Annual Conference on Skeletal Biology and Medicine, The New York Academy of Sciences, New York, USA. Poster Presentation


Scholarships and Awards

2011 Division of Experimental Oncology, Graduate Student Travel Award, University of Western Ontario, London, ON, $1000

2011 Bernier Memorial Award in Skeletal Biology, University of Western Ontario, London, ON, $1000

2009-2011 Canadian Institutes of Health Research (CIHR) Frederick Banting and Charles Best Canada Graduate Scholarship – Doctoral Award, 2 year, $35000/yr

2009 Mogenson Research Scholarship for outstanding research productivity - $1000 cash + $500 travel voucher
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<th>Year</th>
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<tr>
<td>2009</td>
<td>Travel Award, Gordon Research Conference: Bones and Teeth, Biddeford, ME, USA, $500 US</td>
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<tr>
<td>2009</td>
<td>Thesis Research Award Fund, Schulich School of Medicine and Dentistry, Graduate, University of Western Ontario, London, ON, Canada, $950</td>
</tr>
<tr>
<td>2009</td>
<td>Best Poster, Department of Physiology and Pharmacology Research Day, University of Western Ontario, London, ON, Canada, $50</td>
</tr>
<tr>
<td>2008-2009</td>
<td>Ontario Graduate Scholarship Research Award (OGS), Ministry of Training, Colleges and Universities, 1 year, $15000</td>
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<tr>
<td>2006-2009</td>
<td>Canadian Arthritis Network Graduate Award, Canadian Arthritis Declined (2007) Network (CAN), 3 years, $10000/yr</td>
</tr>
<tr>
<td>2006</td>
<td>Travel Award, Canadian Connective Tissue Conference, Ottawa, Canada, $400</td>
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**Other Laboratory Experience**

Co-op Positions

- **2000** Research Assistant, Ministry of the Environment
- **1999** Laboratory Technician, Petro-Canada Research and Development
- **1998** Associate Chemist, Petro-Canada Research and Development, Co-op student
- **1998** Associate Chemist-Sampling, Petro-Canada Research and Development
- **1997** Laboratory Technician, Ontario Clean Water Agency

**Teaching**
Positions

Course Manager
Summer 2010  Physiology 2130 Online (Introductory Human Physiology)

Teaching Assistant
2007-2010  Physiology 1020 Lecture/Online (Intro Human Phys for Nurses)
2006-2009  Physiology 2130 Online (Intro Human Physiology)
Summer 2007  Physiology 550 Lab (Grad Intro Molecular Biology Techniques)
2003-2005  Physiology 130 Tutorial (Introductory Human Physiology)

Awards
2009  George W. Stavraky Teaching Scholarship
2003-2005  Nominated (x2) for a Graduate Teaching Award (GTA)

Supervisory
2010  2 summer students, Shian Yea Wong, Alessandra DiMattia
2009-2010  4th year honours research project, Shian Yea Wong
2009  2 summer students, Shian Yea Wong, Alessandra DiMattia
2006-2007  4th year honours research project, Ashleigh Higgs

University Involvement

Awards
2011 Physiology and Pharmacology Graduate Student Leadership Award, University of Western Ontario, London, ON, $400

Positions

Department of Physiology and Pharmacology, University of Western Ontario:

2011-March Organized, host, and obtained funding for Department Seminar, “Graduate Degrees: Open Doors to Exiting Careers”

2010-2011 Chair of Physiology and Pharmacology Graduate Student Council

2006-2011 Physiology Student Representative at Departmental meeting

2010 Organized the first Physiology and Pharmacology Graduate Student Council (PPGSC)

2008-2010 Graduate Studies Committee

2006-2010 Health and Safety Committee

2008-2009 Mogenson Trust Committee

2003-2005 Society of Graduate Students (SOGS)