October 2015

Epidermal Growth Factor Receptor in Joint Health and Osteoarthritis

Michael A. Pest

The University of Western Ontario

Supervisor
Dr. Frank Beier
The University of Western Ontario

Graduate Program in Pharmacology and Toxicology

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

© Michael A. Pest 2015

Follow this and additional works at: http://ir.lib.uwo.ca/etd

Part of the Musculoskeletal Diseases Commons

Recommended Citation
http://ir.lib.uwo.ca/etd/3277

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca.
EPIDERMAL GROWTH FACTOR RECEPTOR IN JOINT HEALTH AND OSTEOARTHRITIS

(Thesis format: Integrated Article)

by

Michael Andrew Pest

Graduate Program in Pharmacology/Toxicology

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

© Michael Andrew Pest 2015
Abstract

Osteoarthritis (OA) is one of the most prevalent musculoskeletal diseases in the world. Our laboratory has shown that epidermal growth factor receptor (EGFR) signalling is involved in the process of cartilage degeneration in OA. Regulation of EGFR signalling by mitogen-inducible gene 6 (Mig-6) and dual specificity phosphatase 1 (DUSP-1) allows for signal modulation, and mouse models have linked these proteins to joint pathologies. Failure to control EGFR signalling may be involved in OA progression leading to my overarching hypothesis: regulation of EGFR signalling is essential for maintenance of joint health.

I initially tested the role of Mig-6 in cartilage health using cartilage-specific deletion of Mig-6 in a mouse model. Using various histological and imaging techniques, we demonstrated that these animals show increased anabolic activity in articular cartilage, as well as the formation of chondro-osseous nodules in their knee joints within the first 3 months. These mice did not develop severe OA as I predicted, however, there were early signs of developing articular cartilage pathology.

To assess the role of Mig-6 in elderly cartilage, we employed the same mouse model but aged these animals to 21 months, near the end of their life span. These KO animals exhibited similar knee phenotypes, however, no abnormal growths were observed in the ankle and elbow joints which showed enhanced cartilage thickness. Only minor signs of OA were noted. Using an inducible system to delete Mig-6 from cartilage of 3 week old mice, we found limited evidence of increased anabolic activity at 12 weeks. These studies demonstrate that Mig-6 may be playing an important role during development and that loss of Mig-6 may positively impact cartilage health.

Finally, I examined the knee joints of whole body Dusp1 null mice at 21 months of age for signs of OA. Both Dusp1 null and control mice showed similar signs of OA, indicating that DUSP-1 mediated regulation of signalling downstream of EGFR is not essential to prevent spontaneous OA progression.
Taken together, these data demonstrate that EGFR signalling regulated by both Mig-6 and DUSP-1 is important in joint homeostasis, and has revealed a potential target in Mig-6 for future cartilage regenerative treatments.

Keywords

Epidermal growth factor receptor, transforming growth factor alpha, mitogen-inducible gene 6, dual specificity phosphatase 1, p38, ERK, osteoarthritis, articular cartilage, ectopic cartilage, synovial joint, skeleton, Sox9
Co-Authorship Statement


M.A.P. performed most of the experiments and contributed to study design and manuscript writing. B.A.R. performed additional supplementary experiments and contributed to histological processing. Y-W.Z. contributed to editing of manuscript. J-W.J. provided the Mig-6<sup>fl/fl</sup> transgenic mice essential for this study. F.B. contributed to study design and editing of the manuscript. All authors read and approved the submitted version of the manuscript.

**Chapter 3** M.A.P. performed the experiments and contributed to study design and manuscript writing. C.A.P. assisted with processing of mouse gait data. F.B. contributed to study design and editing of the manuscript. These data have not yet been published.

**Chapter 4** is adapted from: Pest MA, Pest CA, Bellini MR, Feng Q, Beier F. Deletion of Dual Specificity Phosphatase 1 Does Not Predispose Mice to Increased Spontaneous Osteoarthritis. Submitted to PLOS ONE.

M.A.P. performed most of the experiments and contributed to study design and manuscript writing. C.A.P. assisted with processing of mouse gait data. M.R.B. assisted with immunostaining experiments. Q.F. provided the *Dusp1* null transgenic mice essential for this study, and contributed to editing of the manuscript. F.B. contributed to study design and editing of the manuscript. All authors read and approved the submitted version of the manuscript.
Acknowledgments

I want to take this opportunity to recognize everyone who has made this thesis possible. Frank, I remember my first few days in the lab very well. I wanted to be as respectful as possible, so I ‘dressed up’ and was always sure to address you as Dr. Beier. I think this phase lasted 3 days tops, and by the end of the week we were on a first name basis and I had settled into my current ratty lab wear. This was probably around the time I timidly asked you for a bit of time off to get married, thanks for granting that request. I have never felt like I was fighting for your time, despite it being split between wrangling numerous students in the lab, a few rambunctious techs, dealing with all those boring PI duties, and then your own kids and grandkids on top of everything (I think you find time for yourself in there somewhere?). Not once have I felt pressured to compromise that essential work/life balance. Your views on how science should be done have deeply impacted the kind of researcher that I think I should strive to be. Your dry wit and humor has comforted me in some of my most difficult times (writing this thesis maybe?). Vielen Dank für alles!

To my supervisory committee, Dr. Peter Chidiac not only did you make a great undergrad thesis supervisor, but your constructive input certainly contributed to this work. Dr. Bob Giffin, your clinical perspective has been invaluable, and Dr. Qingping Feng, you’ve contributed much to this thesis and managed to keep the rest of the committee in line the whole time! It has been a wonderful experience having you all as mentors.

It is said that it takes a whole village to raise a child, and I think that maybe it takes an entire lab to raise a scientist. I am grateful to all of you! Dawn and Holly, bad cop and good cop (not necessarily in that order), I know for a fact that the lab would crumble without you two propping it up. Dawn, I can’t count the number of times I’ve asked you for advice in passing and then come back to my desk an hour or two later to find it stacked with piles of resources with the answer and more. Holly, I can’t count the number of times I’ve asked you for advice in passing and you’ve immediately told me to scram, thanks for that. I remember fearing you almost my entire first year, but now I know you’re just a big teddy bear at heart (the kind that rips hunters to shreds). Seriously though, venting during coffee
breaks with both of you and Jason has been the source of many fond lab memories, and more than a few inappropriate conversations!

As a doctoral student I’ve seen many students come and go, I’ve met a lot of people, and I’ve made a lot of new friends. The first person I had the pleasure of working with was Shirine, who immediately put me to work on skeletal preps (there’s nothing like digging into a mouse skeleton on your first day). Thank you for bequeathing parts of your project to me, it’s been a lot of fun! To the many others who have come and gone from the lab, thanks for making it a fun place to work. Ryan G and Ryan P, thank you for the many stimulating conversations both science and otherwise. To the chipper trio, Sadia, Chantel and Emily, you ladies always had a way of lighting up the room with your bright attitudes. I still smile every time I think of that distinctive Emily laugh that echoed through the halls of LG. Lauren, you’re still one of the most knowledgeable people I know and still are the go to for many of my lab queries, I appreciate your help and friendship during this process. Sara, we were both there in the beginning, shocking everyone with our sudden appearance. I think they took a liking to us quickly though. Thanks for your friendship those first years. Katie, you are the perfect mix of a kind and cheerful individual, who takes absolutely no guff from anyone. I think you’re great, and you will make a fantastic doctor! Jason, you played the role of the cool Post-Doc so well, but that’s just like my opinion, man. I won’t ever forget your advice. Bailey, thanks for being my lab servant for a while. Your contributions to this thesis are tangible (just keep reading!) and I’m very grateful for your help. To the current Beier Brigade (Beier Bunch, I can’t remember what we settled on), you guys are awesome. Paxton, thanks for always making me look good by coming in at noon. You are a brilliant and funny guy, and I know you will kill it as an MD (please not literally). Anusha, not only are you a fantastic person all around, but you are one of the most intelligent and caring people I know, despite all the vile things you have drawn, draped, deposited and poured on my lab bench. You’re one of the best! Margaret, you’ve been around since I can remember (we are the ancients), and it’s been great every minute! Thanks for being a phenomenal benchmate and friend. Melina, I’m still getting to know you, but I am sure you are going to do absolutely amazing things with my project!
Dr. Cheryle Seguin, my kooky lab Aunt, thank you for your mentorship and everything that you have done for both me and my wife, it means more to me than you can know. Matt M, we’ve laughed at many an obscure movie reference, and your friendship has been very important to me. Jake, you always seem cool and calm despite being constantly loaded with caffeine, it’s been a blast. To the rest of the Séguin Squad, if I thanked you all for your friendship and all the things I learned from you it would take a whole extra thesis. Know that I haven’t forgotten any of you. Erik, your wittiness and sarcasm are delightful. Thank you for the endless dorkfest conversations. Kim, I will never figure out how they crammed 7 feet of joy into your itty bitty frame, you are literally one of my favourite people ever. Tom C, you’re one of the most respected people on LG and I know you will continue to shock and amaze everyone with your hilarious wit and pugnacious attitude. To the rest of LG, thank you for your advice, support, friendship and food (potlucks are awesome). I regret missing many of you in these acknowledgements, but let’s be honest, the reader wants to get to the good stuff soon anyway!

I have to thank my family, who have sustained me through all this with absolutely steadfast love and support. Patrick, you are my favourite big little brother, and growing up with you was one of the greatest privileges I will ever experience. Thank you for being a friend and companion for literally as long as I can remember. Mom and Dad, you both instilled values in me that I hold to this day. Your constant encouragement and support are certainly what helped me get this far. Dad, you inspired my curiosity for the world around me and my love of science and inquiry at an early age. If one day I can be half the man, husband, and father you are, I will have achieved greatness. Mom, without your emotional support and guidance I never would have made it this far, knowing that you are always there for me when I need you the most has been the best gift you could ever give me (after the 9 months you spent carrying me that is).

To my burgeoning little London family, I don’t know what I would do without you. My fur baby thesis pets, Lola and Lemon, if you can read this (!!!?) know that you two helped keep me from going crazy through all of it. Courtney, thank you for filling in all the pieces of me that are missing. This life would kill me if I didn’t have you. We’re going to do this together and I can’t imagine a better partner in crime. This is just the beginning.
Table of Contents

Abstract ......................................................................................................................... I
Keywords ....................................................................................................................... II
Co-Authorship Statement ........................................................................................... III
Acknowledgments ....................................................................................................... III
Table of Contents ....................................................................................................... VII
List of Figures ........................................................................................................... XIII
List of Appendices .................................................................................................... XV
List of Abbreviations ............................................................................................... XVI
List of Histological Stains Used .............................................................................. XXI
Chapter 1 .................................................................................................................... 1
  1 Literature Review .................................................................................................. 1
    1.1 The Development of the Skeleton ................................................................... 1
      1.1.1 Intramembranous Ossification ............................................................... 2
      1.1.2 Endochondral Ossification .................................................................... 2
      1.1.3 The Growth Plate .................................................................................... 7
      1.1.4 Secondary Ossification Center ............................................................... 8
      1.1.5 The ‘Bone’ in Bones ............................................................................. 9
      1.1.6 Bone Remodeling .................................................................................. 10
    1.2 Synovial Joints .................................................................................................. 11
      1.2.1 Cartilage .................................................................................................. 12
      1.2.2 Articular Cartilage ................................................................................ 15
      1.2.3 Cartilage Zonation ................................................................................ 16
      1.2.4 Cell Signalling in Chondrocytes ............................................................ 19
    1.3 Arthritis ............................................................................................................. 20
1.3.1 Socioeconomic Burden of Osteoarthritis .............................................. 20
1.3.2 Risk Factors .............................................................................................. 21
1.3.3 Osteoarthritis Pathobiology ...................................................................... 22
1.3.4 Articular Cartilage in Osteoarthritis .......................................................... 23
1.3.5 Subchondral Bone in Osteoarthritis ......................................................... 24
1.3.6 Synovium in Osteoarthritis ........................................................................ 25
1.3.7 Diagnosis of Arthritis .................................................................................. 25
1.3.8 Treatment Methods ..................................................................................... 26
1.4 The Molecular Mechanisms of Osteoarthritis ................................................. 27
  1.4.1 Catabolic Processes ..................................................................................... 27
  1.4.2 Anabolic Processes ..................................................................................... 30
1.5 Cartilaginous Tumours ................................................................................... 32
1.6 Signalling Pathways in Joint Diseases ............................................................. 33
  1.6.1 Epidermal Growth Factor Receptor ............................................................ 33
  1.6.2 EGFR Signaling in Osteoarthritis ............................................................... 34
  1.6.3 Negative Regulation of EGFR Signalling: Mig-6 ........................................ 37
  1.6.4 Mig-6 in Joint Pathology ............................................................................ 38
  1.6.5 DUSP-1 in Joint Pathology ....................................................................... 38
1.7 Animal Models in OA .................................................................................... 39
1.8 Overall Objectives and Hypotheses ............................................................... 40
  1.8.1 Mig-6 in Joint Homeostasis ....................................................................... 40
  1.8.2 Mig-6 in Aging and Joint Maintenance ...................................................... 41
  1.8.3 DUSP-1 in Osteoarthritis ........................................................................ 42
1.9 References ...................................................................................................... 43

Chapter 2 ............................................................................................................. 58
2 Disturbed Cartilage and Joint Homeostasis Resulting From a Loss of Mitogen-Inducible Gene 6 in a Mouse Model of Joint Dysfunction ........................................... 58

2.1 Abstract ......................................................................................................................... 58

2.2 Introduction ..................................................................................................................... 59

2.3 Materials and Methods ................................................................................................. 61

2.3.1 Animals ....................................................................................................................... 61

2.3.2 Skeletal Preparation and Long Bone Measurement ................................................. 61

2.3.3 Histologic Assessment ............................................................................................... 62

2.3.4 Articular Cartilage Measures .................................................................................... 63

2.3.5 Micro-computed Tomography (micro-CT) ................................................................. 63

2.3.6 Statistical Analysis ..................................................................................................... 63

2.4 Results ............................................................................................................................. 63

2.4.1 Normal early bone development in cartilage-specific Mig-6–KO mice... 63

2.4.2 Anabolic buildup of articular cartilage in KO mice ................................................. 67

2.4.3 Formation of chondro-osseous nodules upon cartilage-specific deletion of Mig-6 .......................................................................................................................... 71

2.4.4 Erosion of bone at ligament entheses in KO mice ................................................. 81

2.5 Discussion ....................................................................................................................... 81

2.6 Acknowledgements ......................................................................................................... 88

2.7 Supplementary Tables .................................................................................................. 89

2.8 Supplementary Figures ................................................................................................. 91

2.9 References ...................................................................................................................... 97

Chapter 3 ............................................................................................................................. 102

3 Mice with Cartilage Specific Deletion of Mig-6 Maintain Enhanced Articular Cartilage Thickness into Late Life ................................................................. 102

3.1 Abstract ......................................................................................................................... 102
3.2 Introduction ........................................................................................................... 103
3.3 Methods .................................................................................................................. 105
  3.3.1 Animals ............................................................................................................. 105
  3.3.2 Post-natal induction of recombination ................................................................. 109
  3.3.3 Histology .......................................................................................................... 109
  3.3.4 Micro-computed Tomography (microCT) .......................................................... 109
  3.3.5 Articular Cartilage Thickness and Degeneration ................................................. 109
  3.3.6 CatWalk Gait Analysis ....................................................................................... 110
  3.3.7 Statistical Analysis ............................................................................................ 110
3.4 Results .................................................................................................................... 110
  3.4.1 Mig-6 KO animals are relatively healthy ............................................................... 110
  3.4.2 Calcified nodule formation in 21 month-old Mig-6 KO mice is restricted to knee joint and upper spine ................................................................. 111
  3.4.3 Ectopic nodules are composed of cells that express Cre ................................... 119
  3.4.4 TGFα is not required for ectopic nodule formation in Mig-6 KO mice .......... 119
  3.4.5 Thicker articular cartilage is present in multiple joints of 21 month old Mig-6 KO ................................................................................................................. 123
  3.4.6 Post-natal induction of Mig-6 deletion targets a subpopulation of articular chondrocytes ........................................................................................................... 131
  3.4.7 Post-natal induction of Mig-6 deletion increases lateral tibia articular cartilage thickness ......................................................................................................... 131
3.5 Discussion .............................................................................................................. 132
3.6 Acknowledgements ................................................................................................. 140
3.7 Supplementary Figures ......................................................................................... 141
3.8 References ............................................................................................................ 153
Chapter 4 .................................................................................................................... 158
4 Deletion Of Dual Specificity Phosphatase 1 Does Not Predispose Mice To Increased Spontaneous Osteoarthritis ................................................................. 158

4.1 Abstract ........................................................................................................ 158

4.2 Introduction .................................................................................................. 159

4.3 Methods ....................................................................................................... 161

4.3.1 Animals .................................................................................................... 161

4.3.2 Histology .................................................................................................. 161

4.3.3 Articular Cartilage Evaluation ................................................................. 162

4.3.4 Immunohistochemistry ............................................................................ 162

4.3.5 CatWalk Gait Analysis ............................................................................. 163

4.3.6 Statistical Analysis .................................................................................. 163

4.4 Results ......................................................................................................... 163

4.4.1 DUSP-1 KO animals show good general health up to 21 months of age 163

4.4.2 DUSP-1 KO animals are not protected from the histological changes associated with spontaneous OA ................................................................. 164

4.4.3 DUSP-1 KO mice show similar levels of cartilage breakdown products 164

4.4.4 Loss of DUSP-1 causes an imbalance in anabolic but not catabolic chondrocyte activity ................................................................. 169

4.4.5 The gait of DUSP-1 KO mice is comparable to WT mice ....................... 173

4.5 Discussion .................................................................................................... 179

4.6 Acknowledgements ...................................................................................... 181

4.7 Supplementary Tables ................................................................................ 183

4.8 Supplementary Figures .............................................................................. 185

4.9 References ................................................................................................... 189

Chapter 5 ........................................................................................................... 194

5 Discussion ...................................................................................................... 194
5.1 Overview .................................................................................................................. 194

5.2 Contributions and Significance of Findings ...................................................... 199
   5.2.1 Contributions to the Field of Skeletal Biology ............................................. 199
   5.2.2 Contribution to the Field of Osteoarthritis ................................................. 202

5.3 Limitations of Research ....................................................................................... 203
   5.3.1 Limitations of in vivo models ..................................................................... 203

5.4 Future Directions ............................................................................................... 207

5.5 References ........................................................................................................ 210

Appendices ................................................................................................................. 217
   Appendix A: Animal Use Protocol ....................................................................... 217
   Appendix B: Permission to Include Published Manuscript ............................... 218

Curriculum Vitae ............................................................................................................. 219
List of Figures

Figure 1.1 The Growth Plate ........................................................................................................ 6

Figure 1.2 Diagram of a healthy and osteoarthritic joint (knee) ............................................ 14

Figure 1.3 Articular cartilage organization .............................................................................. 18

Figure 1.4 Epidermal growth factor receptor activation and signalling pathways .............. 36

Figure 2.1 Normal skeletal development in cartilage-specific mitogen gene 6 (Mig-6) knockout (KO) mice and control mice ................................................................. 66

Figure 2.2 Histologic evaluation of knee articular cartilage in 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice . 70

Figure 2.3 Staining for anabolic/catabolic markers in the knees of 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice ........................................................................................................................................ 74

Figure 2.4 Calcified nodules in the knee and spine of 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice........ 76

Figure 2.5 Histology of nodular growths in 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice ................................................. 80

Figure 2.6 Bone erosion in the knee joints of female 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice ................................. 84

Figure 3.1 Experimental design and evaluation of joints ......................................................... 108

Figure 3.2 Extensive formation of chondro-osseous nodules in the knees and spine of 21 month old cartilage specific Mig-6 KO mice .............................................................. 114

Figure 3.3 Ectopic chondro-osseous tissue forms at the base of the skull and the rib joints of 21 month old Mig-6 KO mice ................................................................. 116
Figure 3.4  Ectopic chondro-osseous nodules form in the knee of 21 month old Mig-6 KO mice

Figure 3.5 Loss of TGFα does not protect 12 week old cartilage specific Mig-6 KO mice from ectopic tissue formation in the knee

Figure 3.6 Articular cartilage thickness is enhanced in the knee joints of 21 month old cartilage specific Mig-6 KO mice

Figure 3.7 Articular cartilage thickness is enhanced in the ankle, and elbow of 21 month old cartilage specific Mig-6 KO mice

Figure 3.8 Post-natal deletion of Mig-6 at 3 weeks mildly increased articular cartilage thickness in the knee of 12 week old mice

Figure 3.9 Post-natal deletion of Mig-6 at 3 weeks did not increase the articular cartilage thickness in the elbow of 12 week old mice

Figure 4.1 Dusp1 KO mice show similar cartilage damage as controls at 21 months of age

Figure 4.2 Dusp1 KO mice show similar articular cartilage thickness when compared to controls

Figure 4.3 Cartilage matrix neoepitope immunostaining shows similar intensity and localization in Dusp1 KO and WT control mice

Figure 4.4 Decrease in cartilage anabolism marker SOX9 in Dusp1 KO mice

Figure 4.5 Gait patterns are not different in Dusp1 KO mice at 21 months of age

Figure 5.1 Regulation of EGFR signalling in joint homeostasis
List of Appendices

Appendix A: Animal Use Protocols.................................................................217

Appendix B: Permission to Include Published Manuscript........................218
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACL</td>
<td>Anterior cruciate ligament</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A disintegrin and metalloproteinase with thrombospondin motifs</td>
</tr>
<tr>
<td>Age1</td>
<td>Aggrecan gene</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>ALK</td>
<td>Activin like kinase</td>
</tr>
<tr>
<td>AR</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>BTC</td>
<td>Betacellulin</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMU</td>
<td>Basic multicellular unit</td>
</tr>
<tr>
<td>Calc</td>
<td>Calcaneous</td>
</tr>
<tr>
<td>C1,2C</td>
<td>Collagen I and II cleavage neoepitope</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen induced arthritis</td>
</tr>
<tr>
<td>Col2a1</td>
<td>Type II collagen gene</td>
</tr>
<tr>
<td>Col6a4</td>
<td>Type VI collagen gene</td>
</tr>
<tr>
<td>CreER</td>
<td>Col2a1-CreER(T2) Cre-Estrogen Receptor fusion protein</td>
</tr>
<tr>
<td>DDR2</td>
<td>Discoidin domain receptor 2</td>
</tr>
<tr>
<td>Dio2</td>
<td>Deiodinase-2 gene</td>
</tr>
<tr>
<td>DIPEN</td>
<td>Matrix metalloproteinase mediated aggrecan cleavage neoepitope</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EO</td>
<td>Endochondral ossification</td>
</tr>
<tr>
<td>EPGN</td>
<td>Epigen</td>
</tr>
<tr>
<td>EREG</td>
<td>Epiregulin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERRFI1</td>
<td>ERBB receptor feedback inhibitor 1</td>
</tr>
<tr>
<td>Errfi1</td>
<td>Mig-6 gene</td>
</tr>
<tr>
<td>EXT</td>
<td>Exostosin</td>
</tr>
<tr>
<td>Ext1, 2</td>
<td>Exostosin-1, -2 genes</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FN-f</td>
<td>Fibronectin fragment</td>
</tr>
<tr>
<td>GAG</td>
<td>GAG glycosaminoglycan</td>
</tr>
<tr>
<td>GDF5</td>
<td>Growth/differentiation factor 5</td>
</tr>
<tr>
<td>Gdf5</td>
<td>Growth/differentiation factor 5 gene</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin-binding epidermal growth factor-like growth factor</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulphate proteoglycans</td>
</tr>
<tr>
<td>IHH</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin like growth factor 1</td>
</tr>
<tr>
<td>IGF1R</td>
<td>Insulin like growth factor 1 receptor</td>
</tr>
<tr>
<td>IO</td>
<td>Intramembranous ossification</td>
</tr>
<tr>
<td>JAK-STAT</td>
<td>Janus kinase- signal transducer and activator of transcription</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LFC</td>
<td>Lateral femoral condyle</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>LTP</td>
<td>Lateral tibial plateau</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MFC</td>
<td>Medial femoral condyle</td>
</tr>
<tr>
<td>MIA</td>
<td>Monoiodoacetate</td>
</tr>
<tr>
<td>microCT</td>
<td>Micro computed tomography</td>
</tr>
<tr>
<td>Mig-6</td>
<td>Mitogen-inducible gene 6</td>
</tr>
<tr>
<td>Mig-6</td>
<td>Mig-6 gene (<em>Errf1</em> encodes Mig-6, <em>Mig-6</em> notation used for simplicity)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MKP1</td>
<td>MAP (mitogen activated protein) kinase phosphatase 1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Mmp13</td>
<td>Matrix metalloproteinase 13 gene</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTP</td>
<td>Medial tibial plateau</td>
</tr>
<tr>
<td>Nav</td>
<td>Navicular</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Posterior cruciate ligament</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Prg4</td>
<td>Lubricin gene</td>
</tr>
<tr>
<td>Ptgs2</td>
<td>COX2 gene</td>
</tr>
<tr>
<td>Ptpn11</td>
<td>SHP2 gene</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone related peptide</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RAGE</td>
<td>Advanced glycation end product receptor</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor kappa B</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa B ligand</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2 gene</td>
</tr>
<tr>
<td>SHP2</td>
<td>Src-homology 2 domain-containing phosphatase</td>
</tr>
<tr>
<td>SOX5</td>
<td>SRY (sex determining region Y)-box 5</td>
</tr>
<tr>
<td>SOX6</td>
<td>SRY (sex determining region Y)-box 6</td>
</tr>
<tr>
<td>SOX9</td>
<td>SRY (sex determining region Y)-box 9</td>
</tr>
<tr>
<td>Sox9</td>
<td>SRY (sex determining region Y)-box 9 gene</td>
</tr>
<tr>
<td>Tal</td>
<td>Talus</td>
</tr>
<tr>
<td>TEGE</td>
<td>aggrecanase mediated aggrecan cleavage neoepitope</td>
</tr>
<tr>
<td>TGFα</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>Tgfa</td>
<td>Transforming growth factor alpha gene</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Tib</td>
<td>Tibia</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate resistant acid phosphatase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
# List of Histological Stains Used

<table>
<thead>
<tr>
<th>Stain</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safranin O/Fast Green</td>
<td>Stains red for GAG with a background of green/blue</td>
</tr>
<tr>
<td>Toluidine Blue</td>
<td>Stains deep blue/purple for GAG, blue nuclei, light blue background</td>
</tr>
<tr>
<td>TRAP</td>
<td>Stains purple for tartrate resistant acid phosphatase protein secreted by osteoclasts</td>
</tr>
<tr>
<td>Picrosirius Red</td>
<td>Stains deep red for collagens under unpolarized light.</td>
</tr>
<tr>
<td></td>
<td>Appears red, orange, yellow or green under polarized light</td>
</tr>
<tr>
<td></td>
<td>corresponding to collagen fiber alignment and size.</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>Brown staining indicates antibody binding (targets below)</td>
</tr>
<tr>
<td>COL2</td>
<td>Collagen II, essential cartilage matrix component</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein, used as a Cre reporter</td>
</tr>
<tr>
<td>SOX9</td>
<td>Master chondrocyte phenotype transcription factor</td>
</tr>
<tr>
<td>phERK</td>
<td>Phosphorylated ERK, activated mitogenic protein</td>
</tr>
<tr>
<td>phEGFR</td>
<td>Phosphorylated EGFR, activated receptor</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen, indicates proliferation</td>
</tr>
<tr>
<td>MMP13</td>
<td>Matrix metalloproteinase 13, cartilage catabolic enzyme</td>
</tr>
<tr>
<td>C1,2C</td>
<td>Product of MMP mediated collagen I and II cleavage</td>
</tr>
<tr>
<td>DIPEN</td>
<td>Product of MMP mediated aggrecan cleavage</td>
</tr>
<tr>
<td>NITEGE</td>
<td>Product of aggrecanase mediated aggrecan cleavage</td>
</tr>
</tbody>
</table>
Chapter 1

1 Literature Review

1.1 The Development of the Skeleton

The development of the skeleton marked an important step towards the evolution of mammals. A rigid framework allows for attachment of muscle groups and protection, providing an evolutionary advantage for these early vertebrate creatures [1]. Without a skeletal system, terrestrial life as we know it today would be impossible, as large creatures would collapse under their own weight [1,2]. The skeleton is also highly important in regulation of blood calcium and phosphate, and may also be an important endocrine organ system [3–6]. Modern mammalian skeletons are primarily made up of two different tissue types: bone that forms the framework of the skeleton, and cartilage that enables the bones to interface and form joints and articulate through many degrees of motion. Both of these tissue types share a common progenitor cell of mesenchymal origin [7]. The 206 bones that make up the human skeleton can be roughly classified into 3 categories based on their location: craniofacial (skull), appendicular (limbs), and axial skeleton (vertebra and ribs). The bones that comprise the appendicular and axial skeleton largely form through a process called endochondral ossification, which requires a cartilage template prior to replacement by bone [8]. The craniofacial bones, particularly the flat bones of the skull, form through a process that does not require a cartilage template and is called intramembranous ossification [9]. However, with few exceptions, both processes require the prior recruitment of mesenchymal precursor cells to the location of the future skeletal element. These precursor cells form condensations in a general template of the future bone [10].
1.1.1  Intramembranous Ossification

Only a small number of bones form through intramembranous ossification (IO), including the scapula, pelvis, clavicles, and the flat bones of the skull [9]. In IO, mesenchymal progenitor cells differentiate directly into osteoblasts that produce osteoid the extracellular matrix (ECM) that will then support crystallization of the mineral content that gives bone its rigidity [9,11]. This process is largely driven by the two transcription factors runt-related transcription factor 2 (RUNX2) and downstream osterix [12]. In fact, the mesenchymal precursor cells (also called osteochondral progenitors) of genetically modified mice deficient in either of these factors fail to differentiate into osteoblasts and produce bone, leading to embryonic death of these mice [12].

1.1.2  Endochondral Ossification

The long bones that largely make up the appendicular skeleton (ex. tibia, femur, radius, ulna) form through a process called endochondral ossification (EO) [8,13,14]. While the end result is similar to IO with osteochondral progenitor cells eventually forming mature bone, the long bones must first go through a phase in which a cartilage template is incrementally replaced by bone [8,14]. This cartilage template is formed by cells called chondrocytes that share a common osteochondral progenitor cell origin with osteoblasts [7,14]. Both cell lineages begin with progenitor cells producing the transcription factor SRY (sex determining region Y)-box 9 [SOX9] with concomitant Runx2 expression [7,14]. Cell fate is determined when the osteochondral progenitor commits to either Runx2 expression driving the cell to the osteoblast lineage, or Sox9 which drives chondrogenesis [7]. SOX9 is considered the ‘master regulator’ for the chondrocyte phenotype, and in this role it directly regulates genes that are necessary for chondrocyte function and cartilage
production [15]. Expression of ECM protein genes *Col2a1* and *Agc1* that encode for collagen II and aggregan, respectively, are regulated by SOX9, and are essential for cartilage structure and function [15]. In fact, SOX9 in concert with SOX5/6 (the “Sox Trio”) is essential for the formation and maintenance of permanent cartilage in the skeleton [16].

Following the formation of the mesenchymal condensation, EO proceeds with the differentiation of osteochondral progenitors within the condensation to a chondrocyte lineage, with peripheral cells lining the template forming a perichondrial lining [17]. Perichondrial cells play a role in isolating the growing skeletal element from surrounding mesenchyme environment, and retain the ability to differentiate into chondrocytes or osteoblasts [13,17]. Cells within the mesenchymal condensation proliferate and begin to produce collagen II and aggregan under the control of the Sox Trio [8,13,16]. As the cartilaginous bone template grows through chondrocyte proliferation and matrix production, a zonal structure begins to form along the long axis of bone formation [8]. Cells near the center of the template stop proliferating and grow from a characteristic compact rounded chondrocyte phenotype to an expanded hypertrophic cell phenotype, forming the ‘hypertrophic zone’ [18]. Neighboring cells continue to proliferate and begin to form columns of clonal cells along the axis of bone growth, these columns form the ‘proliferative zone’ [8]. Chondrocytes near the ends of the bone template do not show active cellular proliferation and are more quiescent forming the ‘resting zone’ [8,13]. Collectively these zones form what is called the cartilage growth plate (Fig. 1.1).
Figure 1.1 The Growth Plate The growth plate is involved in development and endochondral ossification, and is organized into distinct zones of chondrocytes. The resting zone near the end of the bone is composed of relatively quiescent chondrocytes with a rounded phenotype. Chondrocytes enter the proliferating zone and form coin-like columns of rapidly proliferating clonal cells. As these cells exit the cell cycle they begin to enlarge and enter the prehypertrophic zone. Terminally differentiated chondrocytes enter the hypertrophic zone and exhibit a large cell volume relative to the resting chondrocytes.
Figure 1.1 The Growth Plate
1.1.3 The Growth Plate

Largely deprived of oxygen and nutrients, chondrocytes in the hypertrophic zone of the growth plate stop proliferating and change programming to produce a unique set of ECM proteins, degradative enzymes, and signalling molecules [13,14]. The production of collagen X rather than collagen II and aggrecan is a hallmark of the hypertrophic chondrocyte phenotype, and is commonly used as a cell marker [14,19,20]. Hypertrophic chondrocytes release vascular endothelial growth factor (VEGF) which recruits vascular invasion into the center of the growth plate [13,14]. Along with nutrients and oxygen, this vasculature brings in osteoclasts that are involved in cartilage template removal so that osteoblasts can lay down new bone matrix [8,14]. This region of new bone formation is called the primary ossification center, which will ultimately expand to form the diaphysis of the mature bone [13,14,17]. Hypertrophic chondrocytes also produce degradative enzymes including matrix metalloproteinase 13 (MMP-13) and aggrecanases that further assist in matrix removal through cleavage of the collagen II and aggrecan network respectively [8,14,21,22]. Ultimately, hypertrophic growth plate chondrocytes will undergo apoptosis, or as recent work suggests, may transdifferentiate to contribute to the osteoblast pool that will replace the removed cartilage matrix with bone [19,20,23,24].

Cells lining the hypertrophic zone differentiate to osteoblasts and contribute to the formation of the cortical bone collar and periosteum. Cells between the hypertrophic zone and the neighboring proliferating zone are called pre-hypertrophic chondrocytes and exhibit an intermediate phenotype between the two zones [13,14].

The proliferative zone is composed of clonal columns of coin shaped proliferating chondrocytes and develops parallel to the direction of bone growth, in a process driven and
regulated by various local and systemic factors [13,15,16]. Along with the expansion caused by the increased cellular volume of hypertrophic cells, this proliferative activity drives the elongation of the bone [13]. Vitamin D, growth hormones and thyroid hormones are all systemic factors that regulate the proliferating zone chondrocytes [25]. However, local growth factors including fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) also play a role [13,26]. The parathyroid hormone related peptide (PTHrP) and Indian hedgehog (IHH) signalling axis permits fine tuning of cellular activity in the proliferative zone and initiation of hypertrophy in the pre-hypertrophic zone [13,14,17,27]. PTHrP produced by both chondrocytes and perichondrial cells at the end of the bone (resting zone) diffuses through the proliferating zone and maintains these cells in a replicative status [13,17]. The PTHrP concentration diminishes farther from the end of the bone and as these chondrocytes exit the cell cycle and enter the pre-hypertrophic zone, they release IHH that has a number of effects on local chondrocytes and the perichondrium [13]. IHH signals back to the end of the bone and maintains PTHrP release, and also induces perichondrial cells to become osteoblasts and form the bone collar [8,13]. This feedback loop helps to maintain zonal organization in the growth plate [13,14].

1.1.4 Secondary Ossification Center

As the bone develops and increases in size, a secondary ossification center forms on either end of the bone and will become the bone epiphysis [14,17]. Similar to the primary ossification center, the secondary ossification center forms when chondrocytes undergo hypertrophy and recruit blood vessels that carry osteoblasts and osteoclasts to lay down new bone [14,17]. The growth plate (now also called the metaphysis) consists of the same zonal arrangement of chondrocytes (Fig. 1.1), sandwiched between the primary
ossification center, and the new secondary ossification center at the end of the bone [13,14,17]. However, a region at the very end of the bone retains chondrocytes that will eventually form the articular cartilage that enables bones to smoothly interface, and articulate with one another in the future joint [14,17]. Closure of the growth plate consists of the slowing and eventual termination of chondrocyte proliferation, with replacement by bone that occurs in humans once skeletal maturity is reached during late puberty [28]. Interestingly, mouse growth plates do not close at sexual maturity, however it seems that high estradiol levels as experienced by humans during late puberty can cause the growth plate to close in mice when delivered exogenously [29].

1.1.5 The ‘Bone’ in Bones

Bones consist of an organ unit made up of various tissues including cartilage, bone tissue, blood vessels, and a marrow cavity filled with hematopoietic stem cells [14,17]. Bone tissue is porous, and contains numerous channels for blood vessels, nerves and the conduction of fluid flow important for transportation of small molecules, nutrients, and information about mechanical loading [30]. The tissue itself consists of a mineralized matrix comprising organic ECM proteins such as collagen I and additional non-collagenous bone matrix proteins [31]. Matricellular proteins regulate the formation of hydroxyapatite crystals that give the bone its rigidity, and may act as signalling molecules during bone remodeling [4,31,32]. There are two general types of bone tissue serving functionally similar but structurally unique purposes. Cortical bone composes the outer regions of all skeletal elements and is densely arranged to provide structural stability and strength to the skeleton [33]. Trabecular bone (also referred to as cancellous or spongy bone) is found near the end of the long bones and in the inner regions of many other bones [33]. This type of
bone is organized in a honeycomb-like network of bone spicules and further supports the cortical bone, while accommodating free space for the marrow cavity [33]. Both types of bone regularly undergo remodeling processes to repair both major and micro fractures, as well as regulating blood mineral homeostasis [4,31,33,34].

1.1.6 Bone Remodeling

Bone is constantly being broken down and reformed to regulate blood calcium/phosphate homeostasis, repair macro and micro bone fractures, and adapt to environmental challenges [4,30]. Destruction of bone is mediated by hematopoietic stem cell lineage-derived osteoclasts [34]. Osteoclasts form when receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage colony stimulating factor (M-CSF) stimulate precursor cells to fuse and differentiate into multinucleated mature cells [34,35]. Activated osteoclasts bind to the surface of the bone through interactions of their surface integrins with the bone matrix to form a unique physiological structure called the ruffled border [34,35]. The ruffled border vastly increases the surface area in contact with the resorption pit that forms underneath the osteoclast [34,35]. The osteoclast then releases a high concentration of hydrogen ions into the resorption pit, acidifying the local environment and freeing the inorganic mineral content in the bone [34,35]. Additionally, proteinases including cathepsin K and various MMPs such as MMP-9, as well as tartrate resistant acid phosphatase (TRAP) are released that then break down the collagens and other ECM proteins that make up the organic component of the bone [34–36].

Conversely, osteoblasts are responsible for laying down new bone during development and in response to osteoclast-mediated bone resorption [34,36]. These cells are derived from the same mesenchymal progenitor cells as chondrocytes, with RUNX2 driving their
differentiation to mature osteoblasts [7,9,36]. Osteoblasts lay down a collection of ECM proteins consisting primarily of collagen I, but also including other non-collagenous structural proteins and signalling proteins [31,33]. This unmineralized matrix is called osteoid, which through a poorly understood mechanism mineralizes when hydroxyapatite crystals form in association with the bone ECM to form a rigid network [31,33]. Osteoblasts regulate the activity of osteoclasts through the release of RANKL and the soluble receptor activator of nuclear factor kappa B (RANK)-like decoy receptor osteoprotegerin (OPG) [33,34]. Together, osteoclasts and osteoblasts form the basic multicellular unit (BMU) that over the course of weeks progressively breaks down and reforms bone in response to the needs of the organism [34].

1.2 Synovial Joints

Individual skeletal elements are generally connected by joints. There are three varieties of joints that form between at least two apposing bones: cartilaginous joints connect the bones through an interface of cartilage (ex. the intervertebral disc in the spine), fibrous joints are connected via tough fibrous collagen rich tissue (ex. sutures between skull bones), and synovial joints that contain a fluid filled space between the bones (ex. knee, ankle, elbow) [33,37–39]. Synovial joints form through a process involving regulated cell death in the joint interzone that will define the joint location, and is partially mediated by muscle movement during embryonic development of the bone [37,40]. Growth/differentiation factor 5 (GDF5), a member of the BMP superfamily, appears to be particularly important both in the formation of joints and skeletal development in general as mice deficient in GDF5 fail to correctly develop joints in the appropriate location and show stunted limb development [37,41].
Synovial joints function as a unit of many different tissue types acting together. At least two bones apposing one another are supported by and anchor muscles that not only allow for locomotion, but also ensure that joint stability and alignment are maintained [42]. Ligaments and other supportive fibrocartilaginous structures (ex. the menisci in the knee joint) further protect joint alignment, distribute load, and prevent damaging overextension or slipping of the joint [43]. The joint capsule and synovium surround the joint and prevent the synovial fluid contained in the joint space from escaping [43,44]. Perhaps most importantly, a layer of articular cartilage caps the bone and provides near frictionless articulating surfaces for the joints to interface [43,45]. Subchondral bone immediately beneath the articular cartilage supports the overlying tissue both mechanically, and through exchange of nutrients and waste products [43,46,47]. An example of a synovial joint is the knee, and is shown in Fig. 1.2 (left half).

1.2.1 Cartilage
Cartilage in general is a connective tissue that depending on the function required may have very different mechanical and structural attributes. Subtypes of cartilage have been defined largely based on their matrix content and function, and include: elastic, hyaline, and fibro-cartilage [48–50]. Fibrocartilage is found in many supporting joint structures, including the meniscus of the knee and the annulus fibrosis in the intervertebral disc of the spine, and its ECM is made up largely of large collagen I fibers [50]. Elastic cartilage is similarly composed largely of collagens but also contains a high proportion of elastin and can be found in the ear and epiglottis [50]. Hyaline cartilage forms both articular cartilage, and growth plate cartilage and is composed largely of collagen II [49,50].
Figure 1.2 Diagram of a healthy and osteoarthritic joint (knee). This schematic representation shows the effects of osteoarthritis (OA) encompassing the whole joint. Healthy articular cartilage (blue) degenerates and may shed fragments into the synovial fluid (yellow). The synovium (red) secretes lubricating molecules, however in OA it becomes inflamed (synovitis) and releases inflammatory compounds into the joint. Subchondral bone remodeling (stippling), and osteophytes may also develop in OA. Supportive ligaments (central) help keep the joint in alignment, and disruption of these structures may lead to OA. Not shown are the additional supportive ligaments (collateral ligaments, etc.) and menisci that further support the joint and distribute load.
Figure 1.2 Diagram of a healthy and osteoarthritic joint (knee).
1.2.2 Articular Cartilage

The primary function of articular cartilage is to prevent excessive friction and wear, allowing smooth articulation between interfacing surfaces in the joint [43,45]. To perform these duties, cartilage has adapted an interesting structure and composition. Unlike many other tissues in the body, healthy cartilage is completely aneural and avascular, and thus depends largely on diffusion of water into and out of the tissue to transport nutrients and waste [50,51]. Chondrocytes are the only resident cell type, and make up approximately 2% of the volume of human cartilage [51,52]. The bulk of the tissue is composed of a complex network of ECM proteins collagen II, various proteoglycans including aggrecan, and matrix crosslinking proteins [49–51]. Aggrecan is rich in charged glycosaminoglycan (GAG) side chains that highly attract water. This is important for cartilage as water is forced out of the tissue during loading, and the charged GAG molecules attract water back into the tissue when load is released [51,53]. This combined with the structural strength contributed by the collagen network makes cartilage a highly durable tissue [49]. However, the relatively acellular, and completely avascular nature of cartilage severely limits its ability for to repair [43,54]. Furthermore, due to relatively slow turnover of the matrix, collagen in the cartilage may have a half-life of decades, and aggrecan may persist for years, meaning the cartilage present at skeletal maturity is essentially the same throughout life [43,55]. Buildup of advanced glycation end products (AGEs) on matrix proteins through low turnover can alter the function of the ECM, and activate the pro-inflammatory AGE receptor (RAGE) that may be involved in some forms of joint pathology [55].
1.2.3 Cartilage Zonation

Cartilage is an anisotropic tissue, with variations in the organization of the matrix and chondrocyte density tailored to suit the functions required at certain depths [49,51]. See Fig. 1.3 for a descriptive diagram. In fact, the anisotropic organization of collagen in cartilage and bone can be shown by picrosirius red staining using histology, which enhances the birefringent effect of collagen and causes selective illumination of specifically aligned collagen fibers under polarized light microscopy [56,57]. The surface of articular cartilage is known as the superficial zone and is directly exposed to the synovial fluid that bathes the joint space [51]. Chondrocytes present in this zone are flattened relative to chondrocytes in deeper zones and express lubricin, a unique lubricating protein encoded by the Prg4 gene, that decreases the coefficient of friction of the joint surface [51,58]. The collagen matrix in this region is organized parallel to the surface of the joint, with the lowest concentration of aggrecan relative to deeper zones [51]. The superficial zone possess the greatest tensile strength in the cartilage, which enables it to cope with the shearing and compressive forces experienced during articulation and loading [51]. Below the superficial zone, mid-zone cartilage has a lower cellular density, however the chondrocyte phenotype more closely resembles the typical rounded resting chondrocyte [51,59]. The matrix of the mid-zone has much higher aggrecan content, with collagen fibers in this zone showing less spatial organization relative to the superficial cartilage [51]. Deep zone cartilage and calcified cartilage show the lowest concentration of chondrocytes and collagen content [51]. The deep zone and calcified cartilage are separated by a meandering line of mineralization visible in histology and referred to as the ‘tidemark’ [51]. The proposed purpose of this calcified cartilage layer is to provide an intermediate
**Figure 1.3 Articular cartilage organization.** Articular cartilage is organized into zones with different cellular, structural, and mechanical properties. The superficial zone (SZ) is in direct contact with the synovial fluid, and consists of collagen fibrils and elongated chondrocytes running parallel to the articular cartilage surface. The mid-zone (MZ) is populated by chondrocytes in a more typical spherical phenotype at a lower density relative to the SZ. Collagen fibrils in the MZ are more randomly organized than the SZ. The deep-zone (DZ) consists of chondrocytes in a roughly columnar organization, with collagen organized roughly perpendicular to the surface and entering the underlying calcified cartilage (CZ). The tidemark (TM) forms a visible demarkation point between the calcified cartilage (CZ) and overlying non-calcified cartilage. Chondrocytes in the CZ are enlarged, and exhibit a hypertrophic-like phenotype. Proteoglycan content (largely aggrecan) increases from the SZ to the DZ and is indicated by the red shading.
Figure 1.3 Articular cartilage organization
region of tissue stiffness between the soft overlying non-calcified cartilage, and the relatively stiff structure of the underlying subchondral bone, thus preventing the cartilage from shearing away [51,60]. Increasing aggrecan concentration in deeper zones of cartilage ensures that water is electrostatically drawn back into the tissue following loading, allowing the tissue to rebound and maintain elastic strength [51].

1.2.4 Cell Signalling in Chondrocytes

Despite the relative isolation of chondrocytes from others cells in the body, signalling readily takes place within the cartilage. Chondrocytes can self-stimulate via autocrine signalling mechanisms, as is seen with epidermal growth factor receptor (EGFR) and release of cell-produced epidermal growth factor (EGF) [61]. Membrane bound factors can also bind their respective receptors on the cell surface through juxtacrine signalling, as has been shown with unprocessed transmembrane transforming growth factor alpha (TGFα) that can also bind EGFR [61,62]. Further juxtacrine signalling pathways include the cell-matrix interactions mediated by integrins, varieties of which can bind a vast array of cartilage ECM proteins including collagen II and fibronectin as well as certain cleaved matrix proteins, and may additionally contribute to chondrocyte mechanosensing of external forces [30,63,64]. Endocrine signalling takes place in articular cartilage, with chondrocytes responding to systemic vitamin D, thyroid hormone, sex hormones and adipokines, among various other hormones [25,65]. In fact, localized inflammation caused by systemic inflammatory factors or those released within the joint may be a driving force for the various diseases that can detrimentally affect cartilage and other joint tissues [59,66].
1.3 Arthritis

Diseases of the joint are as old as the foundation of joints themselves, as shown by evidence of forms of arthritis in the fossil records of ancient reptiles and dinosaurs [67,68]. Dysfunction and disease of joints can take many forms, with many associated etiologies and pathologies [69,70]. Arthritis is a disease of the synovial joint characterized by the breakdown of articular cartilage, leading to pain and dysfunction for the afflicted individual [46]. The most prevalent forms of arthritis are rheumatoid arthritis (RA), which is driven by systemic and local inflammatory breakdown of bone and cartilage in the joint, and osteoarthritis (OA), which has been considered a more ‘wear and tear’ disease in the past but is now thought to have a more complex etiology [43,69–71]. This thesis is focused on osteoarthritis.

1.3.1 Socioeconomic Burden of Osteoarthritis

Osteoarthritis is one of the leading cause of disability in the world, with more than 10% of adults exhibiting symptoms of the disease [72,73]. The most well defined risk factor for the disease is aging, with over 60% of men and 70% of women over the age of 70 showing radiographic evidence of knee arthritis [74]. Indeed, this is most likely an underestimate as early and mid-stage OA are very difficult to diagnose, and typical radiographic evidence is not apparent until the end stage of the disease [43,46]. Symptoms of pain and stiffness typically bring attention to the disease, however by this stage serious degenerative processes have set in and treatment methods are currently lacking [72,73]. The symptoms of pain and stiffness bring with them serious issues of disability that limit physical activity and impact the quality of life of affected individuals [69,72]. Disability in the form of movement limitation is present in over 80% of persons with OA, and over 10% will require
outside assistance to perform daily tasks [72]. As the disease is currently incurable and progresses for perhaps decades, the direct costs of treating the disease are immense, accounting for 1 – 2.5% of the gross domestic product for 1st world countries [43,72]. The indirect costs may be nearly 8-fold higher, and consist largely of lost work time due to absenteeism, decreased productivity, early retirement, and premature death [72]. Furthermore, loss of work hours for caregivers attending to disabled individuals may also contribute to economic impact [72].

1.3.2 Risk Factors

Osteoarthritis can be broadly (perhaps crudely) categorized as either: primary (idiopathic) or secondary (post-traumatic) [43,75,76]. The causes of primary OA are not fully understood, however some risk factors for the disease have been established as: increasing age, obesity, sex (female), and genetics. It is uncommon to see primary OA in those under the age of 40, however varieties of the disease do seem to affect younger individuals [43,69]. The influence of genetic factors has also been well established in twin studies, with the heritability of OA in the spine, hip, and hand estimated at over 60% [77,78]. In comparison, heritability of knee OA has been estimated to be lower than 50%, perhaps due to the prevalence of secondary OA in this joint over others [77]. However, retrospective genome-wide association studies (GWAS) and candidate gene studies have uncovered very few associated genes, with those identified playing roles as structural ECM proteins (Col6a4), inflammation-related (Ptgs2), or developmental genes in bone/joint formation (Gdf5, Dio2) [78,79]. Secondary osteoarthritis, on the other hand, has a better understood etiology initiated typically by trauma to the affected joint [43]. Direct impact to the articular cartilage or subchondral bone can cause direct death and dysfunction of chondrocytes, or
cause subchondral bone changes that initiate overlying cartilage damage [75,80]. Damage or laxity within the supporting ligaments (as seen in catastrophic anterior cruciate ligament [ACL] rupture) can disrupt normal joint alignment, leading to increased abnormal loading and subsequent articular cartilage damage [43,75]. It is currently debated as to whether surgical repair of an ACL rupture is medically warranted, as outcomes have generally been poor, however, animal models demonstrate that there may be benefits for a subset of patients [81]. Obesity also seems to be a risk factor for both forms of the disease, with increased weight leading to higher impact forces experienced by articular cartilage, particularly the large weight bearing joints of the knee and hip [43]. However, obesity is also associated with OA in non-weight bearing joints such as the hands, indicating that additional factors like adipokines or systemic inflammation associated with obesity may be involved [43,82,83]. While there may indeed be additional different subtypes of OA with varying etiology and pathobiology, it seems that the disease progresses to a similar end stage characterized by articular cartilage loss, and pathological bone remodeling [43].

1.3.3 Osteoarthritis Pathobiology

Previous thoughts that OA is exclusively a disease of the articular cartilage are likely not correct. It now appears that OA is a disease involving the entire joint, however the tissue that initiates the disease remains debated [43,80,84]. Indeed, the subchondral bone, meniscus, supportive ligaments, synovium, in addition to the articular cartilage, are all affected by the disease process, and contribute to pathology [43]. Please see Fig. 1.2 for a diagram of healthy joint tissue in comparison to an OA afflicted joint.
1.3.4 Articular Cartilage in Osteoarthritis

While tissues of the entire synovial joint do play a role in the disease, the hallmark of OA remains degeneration of the articular cartilage [43,80]. Changes to the structure and composition of the articular cartilage ECM begin early in the disease with the loss of GAG-containing matrix proteins like aggrecan near the surface of the cartilage [43,51,85]. Following loss of GAG content, the collagen network itself begins to break down, resulting in fibrillation of the articular cartilage surface as the superficial cartilage loses integrity [51,59]. As the ECM network loses integrity, larger fissures may appear and extend into the mid and deep zones of the cartilage [51,59,85]. In the final stages of the disease, the articular cartilage may be worn down to the calcified cartilage, and in severe cases the entire articular cartilage layer is lost, leaving subchondral bone in contact with the apposing articular cartilage or bone and resulting in impaired articulation and perhaps the generation of pain [43,51,59]. The chondrocytes in the matrix are not passive observers as the disease progresses, and respond with a complex set of beneficial and detrimental adaptations [43,86,87]. The anabolic response is characterized by increased production of matrix proteins to replace lost ECM, and proliferation to form clonal sets of chondrocytes to replace cells lost to apoptosis or necrosis and to further increase anabolic potential [43,87]. On the degenerative side, chondrocytes may respond to loss of surrounding matrix and biomechanical changes by altering phenotype to emulate hypertrophic chondrocytes found in the developing terminal growth plate (HZ) [87,88]. These hypertrophic chondrocytes produce increased levels of degradative enzymes (MMP13, aggrecanases, and others) that break down the surrounding cartilage and push the chondrocytes further down the catabolic pathway in a positive feedback loop [87]. These changes in phenotype may also alter matrix
production, leading to a net imbalance in anabolic to catabolic activity in the cartilage and subsequent progression of disease [59,87].

1.3.5 Subchondral Bone in Osteoarthritis

Subchondral bone physically supports and nourishes the overlying avascular articular cartilage [43,80]. Changes in subchondral bone properties have been long associated with OA, most notably the occurrence of subchondral bone sclerosis and osteophyte formation at the margin of the joint [46,80]. OA severity has also been linked to invasion of overlying calcified cartilage by vasculature, altering nutrient and oxygen availability to the resident chondrocytes [80,89]. Formation of bone through altered remodeling of the subchondral bone plate and subchondral trabecular bone progresses during OA to produce a stiffer platform for overlying articular cartilage, and some evidence shows that these changes may precede cartilage damage in some cases [80,90]. Stiffness of the underlying bone alters the mechanical forces experienced by the articular cartilage and may lead to cartilage loss [80]. Furthermore, bone formation may disrupt the normal congruency of the joint surface, further altering the forces experienced by the cartilage through destabilization [75,80]. Additionally, localized regions of bone remodeling called subchondral bone cysts have been shown to form in regions associated with cartilage degeneration, and may precede overlying cartilage damage in certain forms of OA [46,80,91,92]. The formation of bony osteophytes at the margin of the joint is thought to be an adaptive process to balance the altered forces experienced by the joint as the articular cartilage thins, partially stabilizing an imbalanced joint [80,93]. However, osteophytes may also be correlated with pain in OA, although there is conflicting evidence of this relationship [46,94,95].
1.3.6 Synovium in Osteoarthritis

Synovial joints are surrounded by a joint capsule that is internally lined with the synovium. The synovium is inhabited by cells called synoviocytes that are classified as either macrophage-like (Type A), or fibroblast-like (Type B) [44,46,96]. Macrophage-like synoviocytes derive from a mononuclear origin and are partially responsible for the uptake of cell debris and waste from the synovial fluid [44,46,96]. On the other hand, fibroblast-like synoviocytes stem from a mesenchymal origin, and are largely responsible for the secretion of molecules into the synovium such as nutrients, and the lubricants hyaluronic acid and lubricin into the synovial fluid [44,46,96]. Synovitis often appears early on in the OA disease process and is characterized by the thickening of the synovium through synoviocyte proliferation, increased vascularity, and infiltration by mononuclear cells [44]. Synovitis may be initiated by a release of inflammatory factors and matrix breakdown products in the joint following an insult as seen in secondary OA, or through poorly understood systemic and local factors in primary OA [46,75,83]. Following initiation of synovitis, the synovium will also release inflammatory factors such as TNFα and IL-1β that further encourage matrix destruction, leading to a positive feedback loop [44,46,83]. Synovitis has been identified in 90% of patients with OA undergoing knee and hip replacement, and 73% of patients with knee OA assessed by MRI [44,46]. Furthermore, synovial thickening and some synovitis biomarkers correlate well with the progression of symptomatic OA [44,46].

1.3.7 Diagnosis of Arthritis

Diagnosis of arthritis is often reliant on the identification of symptoms by patients, who then seek medical attention. Unfortunately, once the symptoms of pain and joint disability
are obvious, substantial disease progression may have occurred that is currently irreversible [43,46]. Identification of OA is often made with the use of 2D x-ray imaging of the offending joint and examination for key bone-centered OA features including: subchondral bone sclerosis, bony osteophyte growths, and joint space narrowing [43,46,80]. Joint space narrowing is considered evidence of articular cartilage loss as the diminished distance between the visible bones indicates thinning of articular cartilage that normally occupies this area and is invisible on an x-ray scan [46,80]. New techniques utilizing magnetic resonance imaging (MRI) with or without contrast agents are now starting to gain acceptance, and have definite advantages over x-ray based techniques as they do not involve the use of ionizing radiation, and can visualize the entire joint including cartilage in 3-dimensions [46,97]. Testable biomarkers for OA detectable in blood or serum have yet to be used reliably in a clinical setting [98,99]. However, research studies are beginning to use biomarkers of cartilage and bone synthesis and breakdown as measures in the testing of OA drugs and establishing pathobiology [99].

1.3.8 Treatment Methods
Currently there are no effective treatment regimens that are successful in slowing, stopping or reversing the progression of OA once symptoms have emerged [43,46]. Instead, lifestyle modification is often the first step in managing OA. Weight loss, reasonable exercise, education and the use of assistive devices (ex. canes, walkers) are the first line of treatment recommendations for individuals exhibiting symptoms of OA [46,100]. Additional pharmacological pain and inflammation management strategies involve the use of acetaminophen, oral or topical non-steroidal anti-inflammatories, and duloxetine [100]. Intra-articular injections of corticosteroids may also be effective in short term treatment of
pain, but should not be considered a long term solution [100]. Alternative therapy strategies including oral glucosamine/chondroitin, acupuncture, electrotherapy and others are not recommended, however it is likely that they do not harm the patient so long as more reliable strategies are also in use [100]. The use of surgical interventions is reserved for severe end stage OA, when more moderate treatment methods have failed to relieve pain and significant joint dysfunction [43,46,100]. Partial or complete replacement of the joint with a prosthesis, or surgical correction of joint mal-alignment through osteotomy may be effective in resolving joint dysfunction, however these procedures are expensive and carry the inherent medical risks of a highly invasive surgical procedure [43,46].

1.4 The Molecular Mechanisms of Osteoarthritis

While the whole joint is involved in OA, cartilage degeneration is the classic hallmark of the disease [43]. Chondrocytes react to a variety of stimuli both chemical and mechanical, and respond through the anabolic formation of ECM proteins, or production of degradative enzymes and inflammatory factors that activate catabolic activity in the surrounding tissue [80,83,87]. While remodeling of the surrounding matrix is important for normal maintenance of cartilage health, an imbalance in the anabolic and catabolic activity of chondrocytes may lead to a net degenerative effect on cartilage and lead to OA [43,87].

1.4.1 Catabolic Processes

Inflammation is not typically considered a major feature of OA when compared to RA, in which systemic inflammation plays a much more dramatic role [83,86]. However, inflammation in the synovium (synovitis) and cartilage plays a key role in the progression of cartilage degeneration in OA [83]. While many factors are involved, TNFα and IL-1β
appear to be predominantly responsible for the induction of catabolic factors in cartilage degeneration and OA [83,101]. Detectable levels of TNFα and IL-1β can be found in samples of the synovium, synovial fluid, cartilage and subchondral bone taken from the joints of OA patients [83]. TNFα and IL-1β induce the production of proteolytic enzymes that break down the ECM, and encourage the release of additional inflammatory factors including nitric oxide, prostaglandins, and interleukins-6, -17, and -18 [86]. Inflammatory cytokines and adipokines released systemically from white adipose tissue deposits, or locally from the fat pads located within many joints, may also influence cartilage degeneration directly through catabolic enzyme induction, or indirectly by inducing production of other cytokines by synoviocytes and chondrocytes [83,86,102]. Inflammatory cytokines tend to both decrease the anabolic, and increase the catabolic activity of chondrocytes often tipping the homeostatic balance towards loss of ECM [83,86].

Degradative enzymes are released by chondrocytes in response to chemical and physical cues from their environment, and play an important role in physiological cartilage turnover [49,86]. However, excessive production can lead to cartilage breakdown and diseases like OA [59,86]. Currently, 24 mammalian MMP gene products have been identified encoding a wide range of catabolically active enzymes [172]. Generally, MMPs are expressed as inactive proMMPs that must be activated through disruption of a cysteine - zinc ion pro-domain at the catalytic site of the enzyme [172]. Additional regulation at the level of cellular localization further tunes MMP activity, as many of these enzymes are membrane bound [87, 172]. Chondrocytes produce MMPs that are capable of cleaving both collagenous and non-collagenous proteins that make up the ECM network [87]. There are
three major types of MMPs identified in cartilage and OA: the collagenases (MMP-1, -8, and -13), the gelatinases (MMP-2, and MMP-9), and stromelysin-1 (MMP-3) [86]. Cleavage of collagen II and aggrecan by MMP-13 appears to play a key role in OA, as MMP-13 deficient knockout (KO) mice are protected from progression of surgically induced OA [103]. However, while MMP-13 is often considered the primary proteolytic enzyme of OA, areas of degraded cartilage also co-localize with MMP-1, MMP-3, and MMP-8 and others [59,86]. Tissue inhibitor of metalloproteinases (TIMP) may counteract some of the activity of MMPs by binding and inhibiting the active proteinases [86,104]. This is supported by the phenotype of TIMP-3 KO mice that develop OA pathology similar to Mmp13 overexpressing mice [86,105,106].

While MMP-13 has the ability to cleave aggrecan in the cartilage ECM, a family of proteinases collectively called aggrecanases are specialized for proteoglycan breakdown and have been associated with cartilage degeneration in OA [86,107]. Aggrecanase-1 and -2, also known as a disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS-4, -5) respectively, have been implicated as the primary aggrecanases in cartilage [86,107]. However, genetic KO of ADAMTS-5, but not ADAMTS-4 has been shown to attenuate cartilage degeneration following surgical induction of OA in mice, indicating that ADAMTS-5 may be more important in driving OA related aggrecan depletion [21,108].

Normal physiological turnover of the cartilage is important, and releases important growth factors and other signalling factors bound to the matrix. However, breakdown of cartilage may create a positive feedback cycle in which ECM breakdown products, or intact matrix proteins revealed by matrix loss, can incite an inflammatory response, increasing release
of proteinases that further increase the breakdown of the matrix [51,101]. The discoidin domain receptor 2 (DDR2) has been shown to bind native fibrillar collagens I-III with preferential binding for collagen II [109–111]. Breakdown of the matrix constituents surrounding collagen II reveals binding sites for DDR2 and leads to downstream activation of MMP-13 [109–111]. Fibronectin is a component of the ECM that when cleaved by MMPs forms a fibronectin fragment (FN-f) that binds integrins and induces the production of MMP-1 and -3 increasing breakdown of cartilage [63,101]. Synchronized aggregan cleavage by both MMPs and aggreganases forms a 32 amino acid peptide sequence (32mer) that signals to synovial macrophages that then release proinflammatory cytokines IL-1β and TNFα into the joint, further potentiating cartilage breakdown [112,113]. Taken together, these pathways demonstrate that it is important to address OA as early as possible in the disease to prevent positive feedback loops forming from cartilage degeneration.

1.4.2 Anabolic Processes

One of the primary responsibilities of chondrocytes is the production of cartilage ECM during development, and the maintenance of articular cartilage [87]. Growth factors are the key class of signalling molecules that stimulate chondrocytes to produce matrix, and proliferate [114]. Members of the TGFβ superfamily involved in cartilage anabolism and skeletal development include: bone morphogenetic proteins (BMPs), GDF5 and TGFβ itself [114–116]. TGFβ typically binds to a heteromeric complex made up of activin like kinase (ALK) 5 and TGFβ-receptor-II with major downstream targets being SMAD2/3 [114,115,117]. Through this complex, TGFβ promotes chondrocyte production of ECM proteins collagen II and aggregan, and simultaneously down regulates the production of matrix degrading proteinases that has a protective effect in the articular cartilage [114,115].
However, through the SMAD2/3 pathway, TGFβ may also induce subchondral bone remodeling, and synovial fibrosis leading to synovitis [117]. Furthermore, recent studies indicate that TGFβ may switch to a pathogenic ALK1 dominated SMAD1/5/8 pathway in older patients, exacerbating OA related cartilage damage through the induction of chondrocyte hypertrophy [117,118]. BMPs tend to function similarly to TGFβ in chondrocytes by upregulating anabolic genes [114,115]. BMP2 is upregulated in OA chondrocytes by inflammatory molecules TNFα and IL-1β, and seems to partially counteract the catabolic activity elicited by these cytokines [114]. Similarly, BMP7 upregulates anabolic ECM genes and partially mitigates IL-1β mediated inhibition of proteoglycan production [114]. However, as with TGFβ, BMPs may also be involved in OA pathogenesis as SMAD1/5/8 activation by BMPs induces chondrocyte hypertrophy and cartilage degeneration [86,119]. Osteophyte formation induced by both TGFβ and BMP signalling has also been correlated with OA development, further linking these factors to the OA disease process [80,117,118].

FGF18 binds FGF receptor 3 (FGFR3) and may also increase anabolic activity in cartilage through induction of matrix biosynthesis and chondrocyte proliferation [114,115]. In contrast, the effect FGF2 on cartilage seems to be highly context specific, and in some cases can lead to the initiation of chondrocyte proliferation with concomitant induction of catabolic enzyme production, and decreased ECM biosynthesis [114,115]. On the other hand, FGF2 has shown to be effective in vivo in cartilage defect repair, though this may be due in part to increased proliferation of fibroblasts rather than chondrocytes [114]. Insulin-like growth factor-1 (IGF-1) binds to the IGF1 receptor (IGF1R) and plays an important role in the maintenance of cartilage homeostasis by increasing ECM production while
simultaneously inhibiting matrix degeneration [114,120]. While both the IGF-1 ligand and its receptor appear to be highly expressed in OA cartilage, its effects are largely blocked by IGF binding proteins that are also highly expressed in the same OA tissue [114]. However, it appears that IGF-1 further protects cartilage from the catabolic effects induced by IL-1β by upregulating an IL-1 decoy receptor [114,121].

1.5 Cartilaginous Tumours

Anabolic and proliferative activity in chondrocytes may be pathogenic in some bone and joint diseases. Cartilaginous tumors are the most common primary neoplasms found in the skeleton [122,123]. The most common cartilaginous tumors are enchondromas that form within the growth plate and osteochondromas that form adjacent to the growth plate [122,123]. Following an activation event, these tumours may begin to grow and replicate to form larger masses even in adults where the endogenous growth plate has closed [28,122]. Transformation to chondrosarcoma generally occurs during adulthood, and while these malignant tumours are locally aggressive, they rarely appear to metastasize [122]. Osteochondromas typically occur in the long bones in close proximity to the growth plate, however they have been known to appear rarely in the cervical and thoracic region of the spine [124]. Hedgehog signalling, which is important in the regulation of the growth plate, seems to play an important role in chondroma formation [122,123]. IHH upregulates PTHrP production in the perichondrium and inhibits terminal differentiation in the growth plate [13,122,123]. Mutations in the genes that encode proteins exostosin glycosyltransferase 1 and 2 (Ext1, Ext2), which regulate heparan sulphate proteoglycan (HSPG) synthesis, are associated with spontaneous osteochondromas, and result in accumulation of intracellular HSPG [122,125]. HSPG in the ECM bind IHH and thus limit
and regulate its diffusion into the perichondrium and surrounding environment [122,123]. Due to impaired HSPG transport associated with Ext1/2 mutations, abnormal hedgehog signalling in the environment adjacent to the growth plate results in loss of polar organization and permits the growth plate to grow partially in the wrong direction, leading to a benign chondroma growth [122]. Interestingly, recent studies have found that deletion of the gene Ptpn11 that encodes Src-homology 2 domain-containing phosphatase (SHP2), a mediator of EGFR and FGFR signalling, causes metachondromatosis in mice, a disease in which multiple enchondromas and osteochondromas form in the bone [126,127]. These chondromas appear to form via an IHH and FGF2 dependent mechanism, somewhat similar to Ext1/2 mutation related chondromas [122,126,127].

1.6 Signalling Pathways in Joint Diseases
1.6.1 Epidermal Growth Factor Receptor
EGFR is a member of a family of 4 receptor tyrosine kinases (EGFR or ErbB1; Her2 or ErbB2; ErbB3; and ErbB4) located at the cell surface of chondrocytes and many other cell types where they play a role in development, proliferation and differentiation [61,128,129]. Ligands that bind EGFR include: EGF, TGFα, amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epigen (EPGN), and epiregulin (EREG) [61,128,129]. These factors are initially synthesized as transmembrane proteins, and through a process of ectodomain shedding, are proteolytically cleaved by membrane bound metalloproteinases like a disintegrin and metalloproteinas e17 (ADAM17), releasing the EGF-like domain into the environment [61,128,129]. Binding of ligand to EGFR induces formation of homodimers, or heterodimers composed of the 4 EGFR family members, followed by a transphosphorylation reaction (excluding ErbB3 which lacks kinase activity)
and the downstream activation of a number of diverse cell signaling pathways [129].

Downstream signaling pathways include mitogen activated kinase (MAPK) cascades, janus kinase - signal transducer and activator of transcription (JAK-STAT), Akt, and various other protein kinase C (PKC) family proteins [129–131]. These pathways are relatively ubiquitous to many cell types, however the overall downstream biological responses to EGFR ligand binding are cell type- and context-specific [129,132–134].

Cellular changes in response to TGFα mediated EGFR activation include alterations in gene expression and cell proliferation/apoptosis among other metabolic changes [130,135,136]. Please see Fig. 1.4 for a brief synopsis of the main EGFR signalling pathways and ligands.

1.6.2  EGFR Signaling in Osteoarthritis

The EGFR ligand TGFα has been found at increased levels in the joints of a subset of OA patients [137]. Studies have shown that EGFR signaling is involved in the development or protection from OA depending on the context of the OA model [130,137–142]. However, chondrocyte phenotype loss and up-regulation of inflammatory molecules has been observed in response to EGF using in vitro cellular and tissue culture experiments [143,144]. We have shown that TGFα expression in cartilage is increased in a surgical model of OA in rats (transection of the anterior cruciate ligament with partial medial meniscectomy) [139,140]. Additional studies using ex vivo rat osteochondral explants have indicated that TGFα induces a number of catabolic responses including the degradation of ECM proteins collagen II and aggrecan, loss of chondrocyte phenotype and clustering, as well as the induction of OA markers TNFα and MMP-13 [130]. Furthermore, a rat model of surgically induced OA demonstrated decreased progression of cartilage damage when
Figure 1.4 Epidermal growth factor receptor activation and signalling pathways.

Epidermal growth factor receptor (EGFR) is a cell surface receptor tyrosine kinase that signals via many downstream pathways. Receptor activation is initiated by the epidermal growth factor (EGF) family of proteins including: EGF, transforming growth factor alpha (TGFα), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EREG), betacellulin (BTC) and epigen (EPGN). These ligands are found as transmembrane pro-forms that are proteolytically cleaved by cell surface proteases like a disintegrin and metalloproteinase 17 (ADAM17) releasing the EGF-like domain into the environment. Binding of ligand to EGFR causes homodimerization or heterodimerization with other EGFR family receptors (not shown), followed by transphosphorylation of the dimer and subsequent activation of downstream signalling. Cannonical signalling pathways include the PLC-PKC, PI3K-AKT, Ras-Raf-MEK-ERK and Jak-Stat pathways that induce cellular changes as indicated. Regulation of EGFR is mediated in part by Mig-6, which binds to the kinase domain, blocks downstream signalling, and targets the receptor complex for internalization. Additionally, DUSP-1 is involved in dephosphorylization and deactivation of ERK and other MAPK proteins.
an EGFR inhibitor was used [142]. RhoA activation has been demonstrated in response to TGFα stimulation, with following activation of rho-kinase [130]. Pharmacological
inhibition of rho-kinase in a rat osteochondral explant model also showed a decrease in
degradation of collagen II and aggrecan by evaluation of histology with immunostained
ECM breakdown products [130]. Additionally, HB-EGF is increased in the joints of mice
with surgically induced OA, and was found to induce MMPs and inhibit aggrecan
production [64]. These experiments taken together indicate that EGFR signaling may be a
key factor in the process of cartilage degeneration in OA.

1.6.3 Negative Regulation of EGFR Signalling: Mig-6
Cell signalling feedback mechanisms exist to add an additional layer of control to
signalling pathways. The EGFR signalling pathway is under strict regulation to prevent
uncontrolled activation of the various downstream proliferative and angiogenic pathways
that may lead to the development of tumours and cancer, including osteosarcomas
[145,146]. Mitogen inducible gene 6 (Mig-6), which is also known as ERBB Receptor
Feedback Inhibitor 1 (ERRFI1), receptor associated late transducer (RALT) and Gene33
in the literature, is encoded by an immediate early response gene Errfi1 (noted as Mig-6 in
this thesis for simplicity) and has been shown to be involved in regulating overactive
signalling by EGFR (Fig. 1.4) [147]. Down regulation of Mig-6 has been identified in an
EGFR related non-small cell lung cancer, and Mig-6 appears to be involved as a tumour
suppressor in many other cancers [145,148,149]. In fact, cancer cell lines and primary lung
cancer tumours have shown mutations or polymorphisms of Mig-6 [148]. Mig-6 inhibits
EGFR signalling via two primary mechanisms. The activation of EGFR induces
downstream transcription of Mig-6, followed by production of Mig-6 protein that
subsequently binds to the kinase domain of ligand-bound EGFR, thereby inhibiting its
kinase activity and sterically blocking binding partners that facilitate signal propagation
Mig-6 binding also targets active EGFR for desensitization by internalization with subsequent lysosomal digestion [147,151,152]. In cell based experiments, interaction of Mig-6 with additional factors has been identified, including: cAbl, Met, Cdc42 and 14-3-3 proteins [148]. The functional consequences of these interactions has not been clearly demonstrated in vivo, but building evidence indicates that Mig-6 may regulate more than EGFR signalling alone [148].

1.6.4 Mig-6 in Joint Pathology

In addition to increased susceptibility for development of lung adenomas and endometrial cancers, two distinct global Mig-6 mouse knockout (KO) lines have shown progressive joint diseases that result in cartilage degeneration similar to that seen in OA [149,153–156]. Down regulation of Mig-6 has also been observed in a surgical rat model of OA (partial medial meniscectomy) where decreased Mig-6 expression followed induction of OA [157]. In contrast, studies utilizing impact induced OA models in dogs demonstrated an increase in Mig-6 mRNA expression in degenerating cartilage [158,159].

1.6.5 DUSP-1 in Joint Pathology

Many additional factors regulate EGFR signaling both at the receptor level and through modulation of the various downstream signaling pathways. The MEK/ERK pathway is strongly activated by EGFR (Fig. 1.4) and has been implicated in cellular growth and proliferation in cartilage and bone [61,115,130]. EGFR activation by TGFα has been shown to induce activation of both the MEK/ERK and p38 MAPK [130]. Furthermore, c-Jun N-terminal kinases (JNKs) are also activated by EGFR signalling [160,161].
A family of phosphatases collectively referred to as dual-specificity phosphatases (DUSPs) or MAP kinase phosphatases (MKPs) is involved in regulating MAPK signaling. By dephosphorylating MAPK proteins ERK, JNK, and p38, DUSP proteins attenuate signaling by EGFR and other cell surface receptors [162,163]. DUSP-1 (also known as MKP-1) is present in rodent cartilage and may play a role in the chondrotoxicity of certain quinolone antibacterial agents like ofloxacin [164]. DUSP-1 may also play a role in inflammatory rheumatoid arthritis (RA) as increased levels have been detected in fibroblast-like synoviocytes isolated from RA patients [165]. DUSP-1 has also been shown to be a factor in osteolysis and cartilage degeneration in inflammatory models of arthritis [166,167]. However, little is known about the role of DUSP-1 in OA. Inhibition of MEK/ERK signalling using in vitro and in vivo models of OA has been shown to attenuate cartilage breakdown [130,168]. The interaction of DUSP-1 with EGFR downstream signal regulation may play a key role in OA, and thus could be a viable therapeutic target for OA treatment.

1.7 Animal Models in OA

Various models exist that serve to recreate the symptoms and disease process of primary and secondary OA [108,169]. Ideally, human tissues are utilized to maximize the relevance to the medical community. However, healthy control tissues are often difficult to obtain due to ethical issues, and early stage OA samples are equally challenging to identify and acquire. Furthermore, human in vivo studies are often difficult to pursue safely and affordably. Retrospective genome wide association studies (GWAS) have been performed to identify potential genetic factors, although due to the heterogeneity of OA, these studies have been relatively unproductive and have identified few target genes [78,79].
Spontaneous OA has been observed between 16 and 24 months of age in the C57BL/6 mouse strain that forms the background for many of our transgenic mice lines and is thus heavily used to assess the role of various genes in OA [108,170,171]. Combined with ex vivo and in vitro tissue experiments, animal models are powerful tools for evaluating the pathogenesis of OA [108].

1.8 Overall Objectives and Hypotheses

Epidermal growth factor receptor (EGFR) signalling has been implicated in various cancers, and plays a complex role in cartilage degeneration and osteoarthritis as shown by our laboratory. Studies conducted both in vitro and in vivo confirm that EGFR signalling in response to epidermal growth factor (EGF) family ligands leads to significant joint pathology. Based on these studies, my general hypothesis is that regulation of EGFR signalling is essential for maintenance of joint health.

1.8.1 Mig-6 in Joint Homeostasis

1.8.1.1 Objective #1

To characterize the role of Mig-6 in the maintenance of cartilage health using a cartilage specific Mig-6 null mouse model.

1.8.1.2 Rationale #1

EGFR signalling activated by various EGF ligands leads to cartilage degeneration and progression of osteoarthritis. Furthermore, regulation of EGFR by Mig-6 in the entire synovial joint appears to be important in maintaining joint homeostasis, with loss leading to joint disruption and cartilage degeneration. Utilizing cartilage-specific deletion of Mig-6 in mice, I will evaluate the role of Mig-6 in maintaining cartilage health and prevention
of osteoarthritis. These studies may provide additional mechanistic information about osteoarthritis, and reveal new therapeutic targets for its treatment.

1.8.1.3 Hypothesis #1
Loss of Mig-6 in the cartilage of mice will result in excessive EGFR signalling and cartilage degeneration.

1.8.2 Mig-6 in Aging and Joint Maintenance
1.8.2.1 Objective #2
To characterize the role of Mig-6 in aging cartilage, and post-natal regulation of cartilage health.

1.8.2.2 Rationale #2
Mice with whole body deletion of Mig-6 rapidly develop joint pathology and may die prematurely due to the secondary effects of joint immobilization, and thus long term studies have not been conducted. Furthermore, the role of Mig-6 in post-natal cartilage isolated from its effects during development has not been assessed. Utilizing chemically inducible and constitutive cartilage-specific deletion of Mig-6 in mice, I will assess the role of Mig-6 in regulation of homeostasis in post-natal tissue, and in elderly cartilage respectively.

1.8.2.3 Hypothesis #2
Constitutive loss of Mig-6 in cartilage will induce severe osteoarthritis in elderly mice, and inducible deletion in post-natal tissue will lead to premature cartilage degeneration.
1.8.3  DUSP-1 in Osteoarthritis

1.8.3.1 Objective #3

To evaluate the role of DUSP-1 in the regulation of MAPK signalling in osteoarthritis.

1.8.3.2 Rationale #3

EGFR signals via a diverse range of downstream pathways including the various mitogen activated protein kinase (MAPK) signalling cascades. MAPK proteins have been shown to play a role in the regulation of chondrocyte proliferation, apoptosis, and matrix production and turnover in the maintenance of cartilage health, and in disease. Dual specificity phosphatase 1 (DUSP-1) attenuates MAPK signalling and may play a role in regulation of signalling downstream of EGFR in OA. Furthermore, DUSP-1 appears to be important in regulating cartilage and bone degeneration in inflammatory arthritis. Utilizing a whole body Dusp1 null mouse line, I will characterize the role of DUSP-1 in spontaneous age related osteoarthritis and cartilage health.

1.8.3.3 Hypothesis #3

Dusp1 null mice will develop increased incidence and severity of spontaneous osteoarthritis.
1.9 References


64. Long DL, Ulici V, Chubinskaya S, Loeser RF. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is increased in osteoarthritis and regulates chondrocyte catabolic and anabolic activities. Osteoarthr Cartil. Elsevier Ltd; 2015; doi:10.1016/j.joca.2015.04.019


Chapter 2

2 Disturbed Cartilage and Joint Homeostasis Resulting From a Loss of Mitogen-Inducible Gene 6 in a Mouse Model of Joint Dysfunction

This chapter has been adapted from:


2.1 Abstract

Objective: Mitogen-inducible gene 6 (Mig-6) regulates epidermal growth factor receptor (EGFR) signaling in synovial joint tissues. Whole-body knockout of the Mig6 gene in mice has been shown to induce osteoarthritis and joint degeneration. To evaluate the role of chondrocytes in this process, Mig-6 was conditionally deleted from Col2a1-expressing cell types in the cartilage of mice.

Methods: Bone and cartilage in the synovial joints of cartilage-specific Mig-6-deleted (knockout [KO]) mice and control littermates were compared. Histologic staining and immunohistochemical analyses were used to evaluate joint pathology as well as the expression of key extracellular matrix and regulatory proteins. Calcified tissue in synovial joints was assessed by micro-computed tomography (micro-CT) and whole-skeleton staining.

Results: Formation of long bones was found to be normal in KO animals. Cartilage thickness and proteoglycan staining of articular cartilage in the knee joints of 12-week-old KO mice were increased as compared to controls, with higher cellularity throughout the tissue. Radiopaque chondro-osseous nodules appeared in the knees of KO animals by 12 weeks of age and progressed to calcified bone–like tissue by 36 weeks of age. Nodules were also observed in the spine of 36-week-old animals. Erosion of bone at ligament entheses was evident by 12 weeks of age, by both histologic and micro-CT assessment.
Conclusion: Mig-6 expression in chondrocytes is important for the maintenance of cartilage and joint homeostasis. Dysregulation of EGFR signaling in chondrocytes results in anabolic activity in cartilage, but erosion of ligament entheses and the formation of ectopic chondro-osseous nodules severely disturb joint physiology.

2.2 Introduction

Osteoarthritis (OA) is a degenerative joint disease that affects more than 10% of the adult population of North America [1]. There are currently no clinically approved disease-modifying treatments for OA. Consequently, patients experience a general loss in their quality of life, with increased health care costs and lost work time causing considerable socioeconomic damage [2]. While the precise cause and pathobiology of this disease are not fully understood, both mechanical and genetic factors play a key role in specific subtypes of OA [2].

The major hallmark of OA is the degeneration of the articular cartilage and the altered phenotype or death of the only cell type in cartilage, chondrocytes [2]. Chondrocytes are essential for maintaining articular cartilage, which is largely composed of a highly complex network of extracellular matrix (ECM) proteins [2]. In an anabolic state, chondrocytes produce and lay down these structural proteins into the surrounding matrix to repair damage and build up the cartilage [2]. In a catabolic state, chondrocytes release factors to turn over surrounding cartilage through proteolytic digestion (e.g., matrix metalloproteinases) and through release of cytokines (e.g., tumor necrosis factor α and interleukin-1β), which induce catabolic activity in surrounding cells and tissues (synovium, bone, meniscus) [2]. In healthy cartilage, anabolic and catabolic processes are in balance, while in OA, this balance is disrupted toward a net increase in catabolic activity [2].

Bone is important in supporting the overlying cartilage but has also been implicated in the initiation and progression of OA [3]. Both sclerosis of subchondral bone and the formation of bony osteophyte outgrowths are common in the mid-to-late stages of OA in response to local growth factors (such as transforming growth factor β [TGFB] and bone morphogenetic protein) [2]. Abnormal geometry of the bone in a joint can affect the distribution of forces and joint stability and can lead to cartilage degeneration [4]. The
ligaments, tendons, and the meniscus in the knee further stabilize the joint and prevent malalignment and overextension. Many animal models of OA exploit the stabilizing function of the ligaments in the knee joint by using surgical techniques to reproducibly induce a destabilized joint, with subsequent development of articular cartilage degeneration and other OA-like phenotypes [5-8].

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that signals through multiple downstream pathways in a ligand- and context-specific manner [9, 10]. EGFR ligands include epidermal growth factor (EGF), TGFα, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), and others [11]. Evidence shows that EGFR signaling is involved in bone and cartilage physiology during both development and homeostasis in adulthood. EGFR signaling induced by EGF and TGFα is highly important in the regulation of endochondral ossification during the formation of bone [9, 12-14]. We have shown that TGFα/EGF can stimulate catabolic activity in chondrocytes in vitro and cartilage degeneration ex vivo [15, 16]. TGFα is also up-regulated in some forms of human OA and rheumatoid arthritis, as well as in animal models of osteoarthritis [15, 17-19]. EGFR signaling regulates the differentiation of osteoclasts, an important cell type in the turnover of bone [20]. Tight regulation of EGFR signaling is therefore required for the maintenance of cartilage and bone health.

Fine tuning of EGFR signaling takes place at multiple levels, from gene transcription to ligand binding [21]. Mitogen-inducible gene 6 (Mig-6), which is also known as ERBB receptor–feedback inhibitor 1, receptor-associated late transducer, and Gene 33, is a scaffold protein that binds to EGFR and impedes downstream signaling while targeting it for internalization and degradation [22, 23]. Pathways downstream of EGFR activation stimulate the induction of Mig6, that then acts as a negative-feedback response and attenuates EGFR signaling [24].

Whole-body deletion of Mig6 in mice results in a complex set of phenotypes, including the degeneration of articular joints, breakdown of articular cartilage observed at a relatively early age, and severely diseased joints in an injury-induced model of OA [8, 25-27]. Mig-6 deletion targeted to the developing mouse limb mesenchyme using the Prx1-Cre driver
line results in the transient anabolic buildup of cartilage and the development of an OA-like phenotype with increasing age [28]. Since Mig-6 is deleted from all mesenchymal limb tissues in these mice, it is unclear whether bone, cartilage, synovium, or multiple tissue types acting together are involved in the development of cartilage degeneration in these knockout (KO) models.

To evaluate the contribution of Mig-6 in the regulation of EGFR activity in cartilage homeostasis and OA, we used a Col2a1 promoter–driven Cre/lox system to selectively delete Mig6 from chondrocytes in mice.

2.3 Materials and Methods

2.3.1 Animals

All mice were bred and housed in accordance with the Animal Care and Use Guidelines of Western University. To conditionally delete Mig6 in chondrocytes, Mig-6fl/fl [26, 28] mice were bred with Col2a1-Cre mice [29-32] to generate knockout (Mig-6fl/flCol2a1-Cre+/−), heterozygous (Mig-6fl/+Col2a1-Cre+/−), and control (Mig-6fl/flCol2a1-Cre−/− or Mig-6fl/+Col2a1-Cre−/−) animals. Mice were weighed prior to killing by CO2 asphyxiation. Genotype frequency was determined by polymerase chain reaction (PCR) analysis using DNA processed from biopsy samples of ear tissue from mice surviving to at least 21 days of age. Genomic excision of Mig-6 was assessed using standard PCR, with primers flanking the loxP-flanked (floxed) region of the gene (exons 2–4) [26]. Gross morphology of the joint was imaged using a Leica EC3 camera and a Leica S6-D microscope.

2.3.2 Skeletal Preparation and Long Bone Measurement

Animals were euthanized, skinned, and eviscerated on postnatal day 40. Overnight fixation in 70% ethanol was followed by 2 days in acetone. Animals were stained in a solution of 0.05% alizarin red, 0.015% Alcian blue, and 5% glacial acetic acid in 70% ethanol for 10–12 days, with clearing of extraskeletal tissues using a graded series of 2–0.5% KOH [31]. Long bones (femur, tibia, humerus, radius, and ulna) were measured using Leica Application Suite software (v3.8.0) on images obtained using a Leica EC3 camera and a Leica S6-D microscope.
2.3.3 Histologic Assessment

Limbs were fixed in 4% paraformaldehyde for 24 hours and decalcified in 5% EDTA in phosphate buffered saline (PBS), pH 7.0, for 10–12 days. Joints were processed and embedded in paraffin in sagittal or frontal orientation, with serial sections taken at a thickness of 5 μm. All sections were deparaffinized in xylene, rehydrated in a graded series of 100–70% ethanol in water, followed by 100% water, and stained as follows. Glycosaminoglycan content and general histology were assessed in sections stained with Safranin O–fast green (0.02% fast green for 30 minutes, 1% acetic acid for 10 seconds, and 1.5% Safranin O for 3 minutes) or toluidine blue (0.04% toluidine blue in 0.2M acetate buffer, pH 4.0, for 10 minutes). Staining of collagen content was performed using picrosirius red (0.1% sirius red in saturated picric acid solution for 60 minutes, with 0.5% acetic acid washes). The size and organization of collagen fibrils were determined using polarized light microscopy [33]). Light intensity and tissue angle (45°) relative to polarizing filter (Leica no. 11505087) and analyzer (Leica no. 11555045) were kept identical between samples.

Following antigen retrieval and blocking with 5% serum in PBS, sections were stained overnight at 4°C by immunohistochemistry using primary antibody for SOX9 (R&D Systems), phospho-EGFR (phosphoTyr-1173; Cell Signaling Technology), type II collagen (Santa Cruz Biotechnology), or proliferating cell nuclear antigen (PCNA; Cell Signaling Technology). Sections were incubated with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Following DAB+ chromogen (Dako Canada) exposure, sections were counterstained with methyl green (0.5% methyl green in 0.1M sodium acetate buffer, pH 4.2, for 5 minutes). All sections were dehydrated in a graded series of 70–100% ethanol in water, followed by 100% xylene, and mounted using xylene-based mounting media. Tartrate-resistant acid phosphatase (TRAP; Sigma Canada) staining was performed on sections of 12-week-old knee joints according to the manufacturer's instructions, with minor alterations (i.e., 60 minutes in Triton X-100). All images were taken with a Leica DFC295 digital camera and a Leica DM1000 microscope.
2.3.4 Articular Cartilage Measures

Knee articular cartilage thickness and chondrocyte cell density were determined from Safranin O–fast green–stained frontal sections by 2 observers (MAP and BAR) who were blinded with regard to the tissue source. Leica Application Suite software (v3.8.0) was used to evaluate the average articular cartilage thickness by measuring from the articular surface to the subchondral bone interface across 3 points in each quadrant of the knee joint (medial and lateral tibia and femur), in 4 sections spanning at least 500 μm. Cell density of knee articular cartilage chondrocytes was determined by counting all lacunae with evidence of nuclear staining in the medial femur/tibia using a centered region of interest measuring 200 μm wide and 100 μm deep from the articular surface.

2.3.5 Micro-computed Tomography (micro-CT)

Whole-body scans of 36-week-old Mig-6–KO mice were conducted using a GE eXplore speCZT micro-CT scanner at a resolution of 50 μ/voxel. High-resolution scans of the left knee of 12-week-old mice were conducted using a GE eXplore RS micro-CT scanner at a resolution of 20 μ/voxel. Mice were evaluated for morphologic changes using GE Healthcare MicroView software (v2.2) to generate 2-dimensional (2-D) maximum intensity projection (MIP) and 3-D isosurface images [34, 35]. Abnormal tissue was manually highlighted in red in Adobe Photoshop CC.

2.3.6 Statistical Analysis

Data are presented as the mean ± SEM. All statistical analyses were performed using GraphPad Prism software (v6.0).

2.4 Results

2.4.1 Normal early bone development in cartilage-specific Mig-6–KO mice

Excision of Mig-6 from the Col2a1-expressing tissues of Mig-60fl/Col2a1-Cre+/− (KO) and Mig-60fl/+Col2a1-Cre+/− (heterozygous) animals was confirmed using PCR amplification for an excision-specific product (433 bp) (Fig. 2.1A). Xiphoid cartilage, meniscus, and ectopic growths (described below) demonstrated excision of Mig-6, which was not seen
Figure 2.1 Normal skeletal development in cartilage-specific mitogen-specific gene 6 (Mig-6)–knockout (KO) mice and control mice. A, Presence of 433-bp polymerase chain reaction product (indicating recombination) only in joint tissues, but not control tissues (such as the liver), from heterozygous (HET) and KO mice as compared with wild-type (WT) control mice. Nod. = nodule (ectopic growth). B, Photograph of representative 28-day-old (postnatal day 28 [P28]) female control and KO mice used in the studies. Bar = 1 cm. C, Observed (Obs.) genotype frequencies of male and female weanling (P21) mice, showing no statistically significant difference from expected (Exp.) values (P = 0.280), by chi-square test. D, Changes in the weights of mice from P21 to P252, showing no statistically significant differences. E, Lengths of long bones obtained from 40-day-old mice, showing no statistically significant differences. Values in D and E are the mean ± SEM. F, Representative alizarin red/Alcian blue–stained skeletons of 40-day-old mice. Bar = 1 cm. G, Alizarin red/Alcian blue–stained knee joints from 40-day-old mice, showing normal joint morphology but abnormal staining of the tissue anterior to the meniscus (arrow) in the KO mouse. Images are representative of 5 or more mice per group. Bar = 1 mm.
Figure 2.1 Normal skeletal development in cartilage-specific mitogen gene 6 (Mig-6) knockout (KO) mice and control mice
in control liver tissue (Fig. 2.1A) or in the brain or heart (results not shown). Mig-6–KO mice appeared outwardly normal and healthy at weaning (Fig. 2.1B). Genotype analyses based on 644 pups in 86 litters demonstrated no evidence of embryonic or postnatal lethality (Fig. 2.1C). The weights of animals were comparable from birth through adulthood (Fig. 2.1D). Measurement of the femur, tibia, humerus, radius, and ulna showed no statistically significant differences in long bone length on postnatal day 40 in female animals (Fig. 2.1E).

Further evaluation of overall skeletal morphology by alizarin red/Alcian blue staining showed no overt differences in the development of the axial or appendicular skeleton on postnatal day 40 in female Mig-6–KO animals (Fig. 2.1F). Despite a lack of overall skeletal phenotype, evidence of early ectopic growth development in knee joint tissue was seen on postnatal day 40 in some female KO animals, as indicated by diffuse alizarin red/Alcian blue staining of tissue from the anterior meniscus (Fig. 2.1G). The majority of male and female KO mice developed ectopic growths in one or both knee joints, with few exceptions (Supp. Table 2.1). Based on these data and on the similar knee joint histopathologic features between the sexes (data not shown), females were chosen for detailed analyses for the remainder of this study.

2.4.2 Anabolic buildup of articular cartilage in KO mice

Proteoglycan staining (Safranin O) of tissue sections appeared to be increased in the articular cartilage of KO mice at 12 weeks, but decreased at 36 weeks (Fig. 2.2A–D). Picrosirius red staining for collagens showed increased staining in the highly cellular areas in the articular cartilage of KO mice (Fig. 2.2E and F). Further examination of picrosirius red–stained sections under polarized light revealed strong red birefringence of collagen fibers in subchondral bone, with weaker green/yellow birefringence of the articular cartilage in control mice (Fig. 2.2G and H). Interestingly, the superficial and mid-zone articular cartilage of KO animals tended to show more red birefringence as compared to that in control mice, particularly at 12 weeks of age. This may indicate that the ECM organization in these areas is altered compared to controls (Fig. 2.2G and H).
Figure 2.2 Histologic evaluation of knee articular cartilage in 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice. A–D, Safranin O–fast green–stained sagittal sections of knee joints from 12-week-old (A and C) and 36-week-old (B and D) mice show large chondro-osseous nodules (nod.) and bone infiltration (arrowhead and arrow) in the KO mice. Images in C and D are higher-magnification views of the boxed areas in A and B, respectively. E–H, Picrosirius red–stained sagittal sections of the knee joints from 12-week-old (E and G) and 36-week-old (F and H) mice show tissue infiltrating into bone (arrowhead and arrow) in the KO mice. Images in G and H are higher-magnification views of the boxed areas in E and F, respectively, under polarized light. Areas between the broken lines mark superficial and mid-zone cartilage. Images are representative of 5 mice per group. Bars = 200 μm. I, The average thickness of the articular cartilage in the lateral femoral condyle (LFC), medial femoral condyle (MFC), lateral tibial plateau (LTP), and medial tibial plateau (MTP) is significantly increased in the 12 week old KO mice. J, Cell density in the medial femoral condyle and medial tibial plateau are significantly increased in the 12 week old KO mice. Values in I and J are the mean ± SEM. *** = P < 0.001; **** = P < 0.0001 by two-way analysis of variance with Bonferroni multiple comparisons test.
Figure 2.2 Histologic evaluation of knee articular cartilage in 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice.
The thickness of the articular cartilage was increased ~1.5-fold in Mig-6–KO mice at 12 weeks of age in all 4 quadrants of the knee joint (Fig. 2.2I). This increase in cartilage thickness in KO mice was reduced at 36 weeks (data not shown). Cellular density was increased in the articular cartilage of the medial tibial plateau and medial femoral condyle of 12-week-old KO mice as compared to control mice (Fig. 2.2J). No abnormalities in cell number, organization, or phenotype were noted in the growth plate of Mig-6–KO animals.

Immunohistochemistry for SOX9, a key regulator of chondrocyte gene expression, showed clear nuclear staining in the expanded articular cartilage of the knee joints of 12-week-old KO animals (Fig. 2.3A). COL2A1 staining in the superficial and mid zone of the knee cartilage from 12-week-old KO animals was similar to that in the controls (Fig. 2.3B). Immunohistochemical staining for phospho-EGFR (Tyr-1173) was increased in the articular cartilage of 12- and 36-week-old KO animals (Fig. 2.3C and D), as expected upon deletion of a negative regulator. Immunostaining for the proliferative marker PCNA in articular cartilage was increased in 12-week-old KO animals (Supp. Fig. 2.1AB). In the growth plate, staining for phospho-EGFR, SOX9, and PCNA (Supp. Fig. 2.1C, PCNA) in KO animals appeared similar to that in the controls. TRAP staining for osteoclasts was increased in the subchondral (Fig. 2.3E) and trabecular bone (Fig. 2.3F) of 12-week-old KO animals.

Additionally, the elbow joints of 36 week old female and male KO animals also demonstrated thickened articular cartilage (Supp. Fig. 2.2A-D), increased cellular density (Supp. Fig. 2.2E), and SOX9 staining (Supp. Fig. 2.2F).

2.4.3 Formation of chondro-osseous nodules upon cartilage-specific deletion of Mig-6

The formation of osteophyte-like chondro-osseous nodules in the knee joints was observed in nearly all skeletally mature KO mice examined (Supp. Table 1). At 4 weeks of age, knee joints from control and KO mice showed no discernible differences by micro-CT (Fig. 2.4A). However, by 12 weeks of age, all female KO mice had developed visible
Figure 2.3 Staining for anabolic/catabolic markers in the knees of 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice. Immunohistochemistry for SOX9 (A), Col2a1 (B), and phosphorylated epidermal growth factor receptor (pEGFR; Tyr-1173) in 12-week-old animals, for pEGFR in 36-week-old animals (D), and for tartrate-resistant acid phosphatase (TRAP) in the subchondral (E) and trabecular (F) bone in 12-week-old animals was performed on joint sections from control (top) and KO (bottom) mice. Images are representative of 5 mice per group. Bars = 100 μm.
Figure 2.3 Staining for anabolic/catabolic markers in the knees of 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice.
Figure 2.4 Calcified nodules in the knee and spine of 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice. Control and KO mice were scanned by micro–computed tomography at the indicated ages. A–C, Sagittal plane maximum-intensity projection (MIP) images (left) and 3-dimensional isosurface images (right) obtained at age 4 weeks (A), 12 weeks (B), and 36 weeks (C), showing ectopic calcified tissue (arrowheads and red manual contrast versus white for bone) and bone erosion (arrow) in the KO mice. D, MIP images of 36-week-old mice. E–G, Three-dimensional isosurface images, showing the presence of calcified material (red manual contrast versus white for bone) in some of the 36-week-old KO animals at the base of the skull (E), as a fusion between the C7 and T1 vertebrae (F), and between the T10 and T11 vertebrae (G). Images are representative of 3 or more mice per group. Bars = 1 mm in A–C and E–G; 1 cm in D.
Figure 2.4 Calcified nodules in the knee and spine of 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice
nODULES IN AT LEAST 1 KNEE JOINT (Supp. Table 1 and Supp. Fig. 2.2A). THESE NODULES APPEARED AS ENLARGED GROWTHS ASSOCIATED WITH THE PATELLA AND PATELLAR TENDON (Supp. Fig.2.2A).

BY 36 WEEKS OF AGE, MOST KO ANIMALS HAD DEVELOPED NODULES IN BOTH KNEES THAT PREVENTED NORMAL AMBULATION (Supp. Table 1); WE WERE UNABLE TO BEND AND EXTEND THESE JOINTS HARVESTED FROM KO MICE, BUT WERE ABLE TO EASILY BEND AND EXTEND THE JOINTS HARVESTED FROM CONTROL MICE.

AT 12 WEEKS OF AGE, MICRO-CT ANALYSES IDENTIFIED CALCIFIED TISSUE IN THE JOINT SPACE OF KO MICE AS A DIFFUSELY RADIOPAQUE MATERIAL (Fig. 2.4B). THIS TISSUE APPEARED TO SHOW LESS INTENSITY THAN BONE AND FILLED THE ANTERIOR JOINT SPACE AND POSTERIOR AREAS OF THE JOINT (Fig. 2.4B). BY 36 WEEKS, THE NODULES HAD DEVELOPED AN INTENSITY SIMILAR TO BONE AND HAD INTEGRATED FULLY WITH THE SURROUNDING JOINT STRUCTURE (Fig. 2.4C).

FURTHER EXAMINATION OF SIMILAR SYNOVIAL JOINTS BY MICRO-CT SHOWED NO EVIDENCE OF CALCIFIED TISSUE IN THE ELBOW, ANKLE, OR TEMPOROMANDIBULAR JOINT (TMJ) (Fig. 2.4D). HOWEVER, GROWTHS ALONG THE SPINE WERE DETECTED IN MULTIPLE ANIMALS (Fig. 2.4E–G AND Supp. Fig. 2.2BC). NODULES AT THE BASE OF THE SKULL WERE OBSERVED IN KO ANIMALS, ONE OF WHICH SEEMED TO INTEGRATE WITH THE TRANSVERSE LIGAMENT OF C1 (ATLAS) (Fig. 2.4E). FUSION OF THE C7 VERTEBRA WITH THE T1 VERTEBRA WAS OBSERVED IN 1 OF THE KO MICE (Fig. 2.4F). ANOTHER KO MOUSE DEVELOPED NODULES EXTENDING LATERALLY FROM THE TRANSVERSE PROCESSES OF T10 AND T11 (Fig. 2.4G).

HISTOLOGICALLY, THE NODULES APPEARED TO BE COMPOSED LARGELY OF HIGHLY CELLULAR CARTILAGINOUS TISSUE AT EARLIER TIME POINTS (12 WEEKS) (Fig. 2.2A), STAINING STRONGLY WITH SAFRANIN O. AS THE ANIMALS AGED, HISTOLOGIC ANALYSIS SHOWED A TRANSITION TO A MIXED CHONDRO-OSSOEOUS TISSUE (36 WEEKS) (Fig. 2.2B AND 2.5A). EXAMINATION OF SECTIONS UNDER POLARIZED LIGHT SHOWED STRONG RED BIREFRINGENCE IN THE MENISCUS THAT TRANSITIONED TO LESS INTENSE BIREFRINGENCE IN THE PROTEOGLYCAN-RICH REGIONS OF THE NODULES (Fig. 2.5B). HOWEVER, CALCIFIED REGIONS OF THE NODULE SHOWED STRONG BIREFRINGENCE SIMILAR TO THAT OF BONE (DATA NOT SHOWN). HIGHLY POSITIVE TRAP STAINING WAS OBSERVED IN THE AREAS BORDERING
Figure 2.5 Histology of nodular growths in 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice. A, Safranin O (SafO)–fast green stained sections of knee joints from a 36-week-old KO mouse with a nodule. Image at the right is a higher-magnification view of the boxed area in the image at the left. B, Polarized light microscopy [pol.] of a picrosirius red (Picro)–stained nodule (nod.) adjacent to the anterior meniscus (menis.; delineated by the broken line) in section from a 36-week-old KO mouse. C, Tartrate-resistant acid phosphatase (TRAP)–stained nodule in a section from a 12-week-old KO mouse. D–G, Immunohistochemistry for SOX9 (D), Col2a1 (E), and phosphorylated epidermal growth factor receptor (pEGFR; Tyr-1173) in nodule sections from a 12-week-old female KO mouse and for phospho-EGFR in a nodule section from a 36-week-old female KO mouse. Images are representative of 5 mice per group. Bars = 100 μm.
**Figure 2.5** Histology of nodular growths in 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice.
cartilaginous and ossified tissues in the nodular growths from the knees of 12-week-old animals (Fig. 2.5C).

Cells composing the nodules stained strongly for SOX9 (Fig. 2.5D), COL2A1 (Fig. 2.5E), and PCNA (Supp. Fig. 2.1D-F) at 12 weeks. Nodule cells also showed intense staining for phospho-EGFR (Tyr-1173) at both 12 weeks (Fig. 2.5F) and 36 weeks (Fig. 2.5G).

2.4.4 Erosion of bone at ligament entheses in KO mice

Large lesions presenting as radiotransparent noncalcified or partially calcified tissue were visible in the knee joints of Mig-6–KO mice at the age of 12 and 36 weeks, but not at the age of 4 weeks (Fig. 2.6A–D). Histologic analysis revealed that the lesions were associated with ligament entheses, particularly of the anterior and posterior cruciate ligaments (Fig. 2.6E). In the anterior portion of the knee joint, highly cellular, fibrocartilage-like tissue with poor staining for glycosaminoglycan content appeared to invade the subchondral bone via the ligament entheses and did not seem to originate from the articular cartilage (Fig. 2.2A, B, E, and F and Fig. 2.6E). Consistent with these observations, TRAP staining also appeared to be increased in the areas of erosion associated with the ligament entheses (Fig. 2.6F). The growth plate may also be involved in nodular growths and erosion of bone, as invading tissue was associated with a locally disrupted growth plate in severely affected KO mice (Fig. 2.2B, E, and F).

2.5 Discussion

Data from this study suggest that disruption of Mig-6 function in mouse cartilage leads to a complex mixture of anabolic and catabolic effects in the joints. Mig-6 has been studied extensively for its role as a tumor suppressor in many cancers [36-38], but its role in joint development and homeostasis is not well understood, despite the striking joint phenotypes of mice with ubiquitous or Prx1-Cre–driven Mig-6 deletion [8, 25-28]. In the present study, we selectively deleted Mig-6 from chondrocytes using a Col2a1-driven Cre/lox-transgenic mouse to examine the effects of Mig6 deletion in cartilage. Based on the available information, we believe that all or most of the described phenotype in our KO mice was
Figure 2.6 Bone erosion in the knee joints of female 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice. Control and KO mice were scanned by micro–computed tomography at the indicated ages. A–C, Frontal maximum intensity projection (MIP) images obtained at age 4 weeks (A), 12 weeks (B), and 36 weeks (C), showing erosions at the insertion of the cruciate ligament (arrows) in KO mice. D, Three-dimensional isosurface images of 12-week-old control and KO mice, showing erosion of the femur (arrowheads) in the posterior portion of the knee joint of the KO mouse. E, Toluidine blue–stained frontal knee joint sections from 12-week-old control and KO mice, showing a subchondral cyst (asterisk), bone erosion (arrowheads), and abnormal tissue (arrow) at the insertion of the femoral cruciate ligament in the KO mouse. Images at the bottom are higher-magnification views of the boxed areas in the images at the top. F, Tartrate-resistant acid phosphatase (TRAP)–stained section of ligament enthesis from 12-week-old control and KO mice, showing increased staining in the areas of erosion associated with the ligament entheses (arrowheads). Images are representative of 3 mice per group in A–D and 5 mice per group in E and F. Bars = 1 mm in A–D; 200 μm in E and F.
Figure 2.6 Bone erosion in the knee joints of female 12-week-old and 36-week-old cartilage-specific mitogen-specific gene 6–knockout (KO) mice and control mice.
due to increased EGFR signaling. We cannot, however, exclude the possibility that other cellular pathways (e.g., signaling from other receptor tyrosine kinases) contribute to some degree.

Suppression of cellular proliferation by Mig-6 has previously been shown under multiple experimental conditions in vitro and in vivo [39-41], for example, in keratinocytes [25-27]. Our previous work demonstrated that EGFR activation by TGFα promotes proliferation in cultured chondrocytes [15, 18]. Here, we demonstrated an increase in cellular density in the articular cartilage and high cellularity in chondro-osseous nodules in the cartilage-specific Mig-6–KO animals. Consistent with this finding, the increased staining for PCNA in KO animals points to an increase in proliferation in the articular cartilage. Greater numbers of cells expressing SOX9 and displaying EGFR activation (e.g., phospho-EGFR staining) were present in both the articular cartilage and nodules from KO animals. An increase in SOX9 has also been shown in response to EGFR ligands (amphiregulin, HB-EGF, and others) in human urethral cancer cells [42]. Furthermore, a recent study has demonstrated that Prx1-Cre–driven deletion of Mig-6 causes increased SOX9 and EGFR activation in cartilage as well as increased cell proliferation in the knee joint [28], similar to what we found in the present studies. In contrast, in our in vitro studies, TGFα was found to decrease Sox9 expression [15, 18].

One potential explanation for these seemingly contradictory findings is that our previous studies examined Sox9 messenger RNA levels on a per cell base, whereas here, we studied the expression domain of SOX9 protein in tissue sections. It is therefore feasible to speculate that the increased expression of SOX9 in our Mig-6–KO mice is secondary to increased chondrocyte proliferation, which results from activation of EGFR signaling.

With an increase in chondrocyte number in the articular cartilage of Mig-6–KO animals, an increase in cartilage thickness is not unexpected. Examination of picrosirius red staining under polarized light revealed that the structure of the articular cartilage ECM in KO mice was altered compared to that of control mice. The noncalcified articular cartilage of control animals showed little red-orange birefringence, while that from KO animals showed increased intensity, which may indicate a disruptive shift toward larger, organized collagen
fibers [33]. The resultant altered stiffness of the cartilage may have an impact on the structural integrity of the cartilage [43]. Even so, we were surprised to find that even at 36 weeks of age, the degeneration of articular cartilage from the Mig-6–KO animals was far milder than in previously described whole-body– and limb mesenchyme–targeted KO animals [25, 28]. Increased subchondral bone stiffness can contribute to the degeneration of overlying cartilage by subjecting it to increased load [2, 44], and synovial tissue may also secrete catabolic factors [2]. Normal levels of Mig-6 in the bone and other joint tissues of our animals could explain the lack of articular cartilage damage compared to whole-joint levels in Mig-6–KO mice.

However, the difference in cartilage thickness between KO and control mice was much less pronounced at 36 weeks than at 12 weeks. Since the superficial zone appeared to be structurally intact (although with decreased Safranin O staining), the relative loss of cartilage in older KO mice might be due to an advance of the tidemark and, ultimately, replacement of cartilage by bone through endochondral ossification. This model is supported by increased TRAP staining in subchondral bone, as well as by earlier studies showing that TGFα/EGFR signaling in cartilage promotes RANKL expression, osteoclast recruitment, and cartilage-to-bone transition [12, 13]. Moreover, these data suggest a biphasic mode of EGFR/Mig-6 signaling, where it initially promotes articular cartilage growth through chondrocyte proliferation, but ultimately causes events associated with cartilage maturation and replacement by bone.

Loss of Mig-6 has been shown to induce the production of abnormal skeletal growths. Whole-body KO of Mig-6 leads to the formation of osteochondral nodules in the knee, ankle, and TMJ [25, 26]. Conditional knockout of Mig6 in the limb mesenchyme also results in growths that have been described as central and lateral osteophytes in the knee, although their presence in other joints was not reported [28]. In our study, targeted deletion of Mig6 in chondrocytes resulted in the formation of chondro-osseous nodules primarily in the knee joint. In contrast to previous findings [25, 26], we did not identify abnormal calcified tissue in any other appendicular synovial joints or in the TMJ. Histologic analysis also failed to show abnormal tissue in the elbows of 36-week-old Mig-6–KO animals.
However, calcified nodular growths were identified in the spine of 36-week-old animals, indicating that the nodule phenotype is not restricted to the knees.

In many of the Mig-6–KO animals used in this study, nodules did not form with the same dynamics in both knees. Differences in size or complete absence of nodular formation in one knee occurred in both sexes at 12 weeks of age. However, by 36 weeks, the nodules had grown substantially and were present bilaterally in nearly every animal. Osteophytes, which are similar to these nodules, commonly form as a result of surgical insult to the joint in many rodent models [5-7], although usually not as exaggerated as in this study. Induction of Mig-6 has been shown in canine impact models of OA [45, 46] and induction of TGFα in rat surgical models of OA [18], demonstrating that EGFR signaling is sensitive to joint injury and mechanical stress. This may indicate that an insult to the joint is required to initiate the process of abnormal growth, perhaps in a dysregulated attempt to repair localized damage.

One open question is the cellular origin of the nodules. Since cells in the nodules show high levels of phospho-EGFR, it seems likely that they derive directly from cells in which the Mig-6 gene has been inactivated (e.g., in which type II collagen–Cre is or was expressed). This is further supported by the findings of our PCR analyses of genomic recombination. While it is possible that Col2a1-Cre–mediated Mig-6 deletion during development of the synovium may also be involved [47], the lack of nodules observed in other synovial joints indicates that a structure specific to the knee is most likely the source of this pathologic change. Indeed, many nodules appear to start from the meniscus that expresses Col2a1 during its formation [48].

Novel to this study, cartilage-specific Mig-6–KO mice developed erosive lesions surrounding ligament entheses and exhibited increased TRAP staining in the trabeculae, subchondral bone plate, and cartilaginous tissue–ossified tissue junctions that form in the knee nodules of KO animals. The EGFR pathway has previously been identified as a regulator of osteoclast recruitment to developing bone as a normal component in endochondral ossification [12-14, 20]. Diminished EGFR signaling through deletion of TGFα or pharmacologic inhibition of EGFR results in decreased RANKL expression in
cartilage and decreased TRAP staining in the underlying trabecular bone [12, 13, 49]. However, it is still unclear how osteoclasts would be recruited and sustained at the ligament entheses to create the levels of damage observed at such an early age in our Mig-6–KO animals.

Recent studies described by Staal et al [50] produced data in similar animal models that support the results shown here. Taken together, these studies suggest that a fine balance in EGFR signaling is required to maintain joint homeostasis. Under controlled conditions, therapeutic agents that increase EGFR signaling may be a viable option for promoting anabolic activity in articular cartilage. However, the complications of overstimulation of the EGFR pathway have been demonstrated here, and additional work is required to further elucidate this complex pathway and its effects in cartilage and joint homeostasis.

2.6 Acknowledgements

We would like to thank Holly Dupuis for her assistance with TRAP staining. M.P. is currently funded by a Canadian Graduate Scholarship Doctoral Award from the Canadian Institutes of Health Research (CIHR) and the CIHR Joint Motion Program, and has been previously funded by the Canadian Arthritis Network. This work was supported by a CIHR grant (MOP 86574) to F.B. who is also the recipient of a Canada Research Chair Award.
2.7 Supplementary Tables

**Supplementary Table 1: Number of Mig-6 KO and Control animals with ectopic nodule growths in knee joints** Animals were examined via dissection or through microCT to assess the knee joints for nodules. Incidence of nodules in neither knee, a single knee joint, or both knee joints (in a single animal) are noted.
**Supplementary Table 1**: Number of Mig-6 KO and Control animals with ectopic nodule growths in knee joints

<table>
<thead>
<tr>
<th>Knee Joints/animal with nodule formation</th>
<th>12 Weeks</th>
<th>36 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>KO</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Single</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Both</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Single</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Both</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>
2.8 Supplementary Figures

Supplementary Figure 2.1 Proliferative marker PCNA is detected in Mig-6 KO articular chondrocytes and ectopic growths. (AB) Immunohistochemistry for proliferative marker PCNA (brown) shows increased nuclear staining (arrowheads) in 12 weeks old KO animals (right panels) compared to Controls (left panels). (C) Growth plate shows no difference in PCNA staining. (DE) Strong PCNA staining in (D) chondrocyte-like cells, (E) chondrocyte-like and fibrotic cells of the ectopic growths in KO animals. (F) PCNA staining of cells in the cruciate ligament entheses of KO animals. Representative images shown. N=5. All scale bars = 20μm.
Supplementary Figure 2.1 Proliferative marker PCNA is detected in Mig-6 KO articular chondrocytes and ectopic growths.
Supplementary Figure 2.2 Anabolic growth of elbow cartilage in 36 week old female cartilage specific Mig-6 KO mice. (A) Safranin O/fast green stained sagittal sections of the elbow joint of 36 week old Control (left) and cartilage specific Mig-6 knock out (KO) mice (right). (B) Average cartilage thickness in the humerus, (C) radius and (D) ulna is up to 1.6-fold thicker in both female and male KO mice. (E) Average chondrocyte density in the humerus was increased 1.5-fold in female KO animals compared to Control. (F) Immunohistochemistry for SOX9 shows positive staining in chondrocytes in KO animals. Representative images shown. N=5 in all experiments. Scale bars represent 100 µm. Mean values shown ± SEM. Data analyzed by two-way ANOVA with Bonferroni’s multiple comparison test, ** P<0.01, ***P<0.005, ****P<0.001
Supplementary Figure 2.2 Anabolic growth of elbow cartilage in 36 week old female cartilage specific Mig-6 KO mice
Supplementary Figure 2.3 Chondro-osseous nodules are visible in knee joint and spine of female cartilage specific Mig-6 KO mice. (A) Left knee joints of female Mig-6 knock out (KO) and Control animals at 12 (left) and 36 (right) weeks of age. (B) A nodule is visible in the lateral thoracic spine of a female KO animal. (C) Chondro-osseous nodules are present in the spine (arrow) and knee (arrowhead), but not the ankles or elbows of a 39 week old female animal. Representative images shown. N=5, AB. N=1, C. All scale bars = 1mm.
Supplementary Figure 2.3 Chondro-osseous nodules are visible in knee joint and spine of female cartilage specific Mig-6 KO mice
2.9 References


Chapter 3

3 Mice with Cartilage Specific Deletion of Mig-6 Maintain Enhanced Articular Cartilage Thickness into Late Life

These data are currently unpublished, but will contribute to an original first author research paper to be submitted for publication:

Pest MA, Pest CA, Beier F. Mice with Cartilage Specific Deletion of Mig-6 Maintain Enhanced Articular Cartilage Thickness into Late Life. To be submitted to Arthritis & Rheumatology, 2015

3.1 Abstract

**Background:** Mig-6 acts as a negative regulator for epidermal growth factor receptor (EGFR) signalling. Attenuation of EGFR signalling decreases osteoarthritis (OA) related cartilage degeneration in rodent models, however, cartilage-specific Mig-6 knockout (KO) in cartilage has been shown to induce ectopic chondro-osseous nodule formation without significant cartilage degeneration in young mice. Conversely, whole body Mig-6 deletion causes rapid joint degeneration. We aged cartilage-specific Mig-6 KO mice to evaluate the long term effects of Mig-6 loss, and induced post-natal deletion of Mig-6 in mice to evaluate the role of Mig-6 in joints beyond development.

**Methods:** The synovial joint tissues of the knee, ankle, and elbow were assessed in 21 month-old cartilage specific Mig-6 KO animals using histological stains, and microCT for calcified tissue. Cartilage degeneration was scored and gait changes were also evaluated. Post-natal deletion of Mig-6 in cartilage was induced by tamoxifen with Col2a1-CreER mice at 3 weeks, and followed up at 12 weeks.

**Results:** The articular cartilage of 21 month-old Mig-6 KO animals showed increased thickness in the knee, elbow, and ankle relative to controls. Ectopic chondro-osseous nodules were also identified in the knee, and upper spine. Cartilage degeneration, as shown by glycosaminoglycan (GAG) loss, was evident in the knees of 21 month-old Mig-6 KO animals. Mig-6 Tgfa double KO mice developed similar nodules in the knee joint at 12
weeks of age. When Mig-6 was deleted in 3 week-old animals, and followed up at 12 weeks of age, no major changes in articular cartilage thickness or chondrocyte density were observed.

**Conclusion:** Loss of Mig-6 during development causes anabolic changes in articular cartilage that persist late into the life of mice at 21 months of age, while postnatal Mig-6 deletion produced few observable effects at 12 weeks of age. Further research is needed to evaluate the complex role of Mig-6 and EGFR signalling in cartilage health for potential use as a therapeutic target or in regenerative medicine.

### 3.2 Introduction

Degenerative joint diseases such as osteoarthritis and rheumatoid arthritis pose a serious issue for population health in the US [1]. Costs in the form of primary health care, increased incidence of comorbid diseases, and lost work hours are a significant burden to the economy [1,2]. For osteoarthritis (OA), there are currently no effective means to slow or stop the disease, and only the symptoms of joint pain and stiffness can be addressed. The disease eventually progresses to end-stage where one of the only solutions is joint replacement [2]. As the pathobiology of OA is poorly understood, additional research is needed to elucidate the molecular mechanisms responsible for the initiation and progression of joint degeneration.

Articular joints are composed of multiple different tissue types acting in concert to provide locomotive ability and stability. Articular cartilage caps the bones of the joints, and provides a frictionless surface for the joint surfaces to interface [3]. Subchondral bone supports and nourishes the overlying articular cartilage. Ligaments, muscle and the menisci in the knee prevent joint destabilization and distribute load within the joint [2]. Dysfunction or damage to any one, or all of these tissues may be involved in OA progression, with additional risk factors including increasing age, genetics, and obesity [2].

Articular cartilage degeneration is the accepted hallmark of OA. This layer of extracellular matrix (ECM) rich tissue contains a limited number of chondrocytes, which are the only resident cell type [2]. Chondrocytes are responsible for laying down ECM proteins such as
collagen II and sulfated glycosaminoglycan (GAG) rich proteoglycan molecules (most prominently aggrecan) into a complex network, which gives the cartilage its great elastic flexibility and compressive strength [4]. Matrix turnover is also conducted by chondrocytes, which produce matrix metalloproteinases (MMPs) and aggrecanases to break down collagens and aggrecan as a part of regular maintenance in the tissue [4]. In healthy tissue, the anabolic buildup and catabolic breakdown of cartilage ECM is in balance. However, this may become upset by local inflammatory factors like TNFα and IL-1β that induce excess production of catabolic enzymes, and decrease ECM protein production [3,4]. Growth factors such as TGFβ and BMP partially oppose ECM breakdown by increasing production of matrix proteins, however this is generally insufficient to prevent cartilage breakdown and may contribute to the formation of bony osteophytes at the joint margin [2].

Our lab and others have previously shown that activation of epidermal growth factor receptor (EGFR) by its ligands epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and heparin-binding EGF-like growth factor (HB-EGF) increases cartilage degeneration in vitro and in vivo, and that attenuation of EGFR signalling partially protects animals from progression of surgically induced osteoarthritis [5–9]. However, recent work has revealed that genetic deletion of Mig-6, which encodes the protein Mig-6 that acts as a negative regulator of EGFR signalling, may induce formation of thicker articular cartilage, ectopic chondro-osseous nodules, and increased chondrocyte proliferation [10–13]. Mig-6 is encoded by an immediate early response gene (Errf1, denoted Mig-6 in this text for simplicity) and targets ligand-bound EGFR for internalization, lysosomal degradation, and further blocks receptor transactivation [14]. Beyond its role as a negative-feedback sensor for EGFR, in the absence of EGFR ligands Mig-6 also appears to interact with c-Abl and may be involved in cellular apoptosis [15]. Mice with whole body, or limb mesenchyme-specific deletion of Mig-6 initially show increased articular cartilage thickness, however, within a few months of life they begin to show early signs of cartilage degeneration similar to osteoarthritis, the mechanism for which is still unclear [12,13,16,17]. Conversely, mice with cartilage-specific deletion of Mig-6 appear to develop thicker articular cartilage in the knee that is maintained into adulthood [10,11]. Further research is required to evaluate the role of Mig-6 and EGFR signalling in the anabolic maintenance and development of
articulate cartilage, for possible use as a therapeutic target in OA and other degenerative diseases of cartilage.

In this study, we aged mice with cartilage-specific deletion of Mig-6 to 21 months of age to evaluate the role of Mig-6 in cartilage homeostasis and OA development. Additionally, Mig-6 deletion in chondrocytes was conditionally induced in three week-old mice to assess the role of Mig-6 in postnatal cartilage tissue.

3.3 Methods

3.3.1 Animals

All mice used in this study were bred and housed in accordance with the Animal Care and Use Guidelines of Western University – Canada. Mice were weighed prior to sacrifice by CO₂ asphyxiation. As previously described [10], Mig-6 (gene Errfi1, denoted as Mig-6 for simplicity) was conditionally deleted in chondrocytes through breeding Mig-6<sup>fl/fl</sup> [16] to Col2a1-Cre<sup>+/−</sup> [18] mice creating knockout (KO, Mig-6<sup>0/0</sup>Col2a1-Cre<sup>+/−</sup>) and control (Mig-6<sup>0/0</sup>Col2a1-Cre<sup>+/−</sup> or Mig-6<sup>0/0</sup>Col2a1-Cre<sup>−/−</sup>) mice (Fig. 3.1A). Only female mice were examined for 21 month and 4 week (supplementary data) time points. Additional experiments utilized mice bearing either the ROSA26<sup>mTmG</sup> reporter gene [19] or Tgfa null (encodes TGFα cytokine) mice [20] in concert with Mig-6<sup>0/0</sup> and Col2a1-Cre<sup>+/−</sup> to evaluate cell fate within the articular cartilage and ectopic nodules, and the role of TGFα in ectopic tissue formation respectively.

Post-natal conditional deletion of Mig-6 utilized the same ‘floxed’ Mig-6 line above, bred to a tamoxifen inducible Col2a1-CreERT2 [21] line of mice to produce CreER(+) [Mig-6<sup>0/0</sup>Col2a1-Cre<sup>+/−</sup>] and CreER(−) [Mig-6<sup>0/0</sup>Col2a1-Cre<sup>−/−</sup>] mice (Fig. 3.1B). One additional trial consisted of CreER animals that were heterozygous for the ROSA26<sup>mTmG</sup> reporter gene [19] to evaluate tamoxifen induced Cre activation in articular cartilage chondrocytes. Male mice were used for tamoxifen experiments. Genotyping and assessment of genomic recombination was performed on DNA samples processed from ear and xyphoid cartilage tissue biopsies. Standard PCR was performed using primers on either side of, and within the ‘floxed’ Mig-6 allele (Mig-6 exons 2-4 flanked by loxP sites) to amplify products of the recombined or intact gene [10,16].
Figure 3.1 Experimental design and evaluation of joints. (A) Cartilage specific Mig-6 knockout (KO, Mig-6^{fl/fl}; Col2-Cre) and control mice (Mig-6^{fl/fl} or Mig-6^{fl/+}) were aged to 21 months to evaluate synovial joint integrity and osteoarthritis. (B) Inducible cartilage specific Mig-6 KO mice (Mig-6^{fl/fl}; Col2-CreER) were injected at 3 weeks of age with 5 courses of tamoxifen or vehicle over 5 days. Mice were then aged to 12 weeks of age before sacrifice. (C) Diagrams of the knee, elbow and ankle joint evaluated in this study. Approximate areas of articular cartilage measurements are indicated by dashed red lines.
Figure 3.1 Experimental design and evaluation of joints
3.3.2 Post-natal induction of recombination

*Mig-6* recombination and resultant deletion in 3 week old CreER(+) male mice was induced by intraperitoneal injection of 75 mg/kg tamoxifen dissolved in sterile corn oil delivered in 5 doses over 5 days (Fig. 3.1B). CreER(-) mice were used as controls, with additional controls using sterile corn oil vehicle delivered to CreER(+) and CreER(-) mice. Following the final course of tamoxifen, mice were housed for 5 days in an inclusion facility and then transferred to general housing until sacrifice at 12 weeks of age.

3.3.3 Histology

The knee, elbow, and ankle joints of mice were taken and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.0) for 24 hours at room temperature. The intact joints were then decalcified in 5% ethylenediaminetetraacetic acid (EDTA) in PBS (pH 7.0) for 10 – 12 days at room temperature. All joints were processed for paraffin embedding, and 5 µm thick serial sections were taken in the sagittal or frontal orientation as indicated. Sections were stained with either toluidine blue (representative images shown) or safranin O/fast green to evaluate glycosaminoglycan (GAG) staining, and structural changes in the joints, as previously described [10]. Immunostaining was performed using anti-GFP antibody (Cell Signalling) and protocols previously described [10]. Images were taken using a Leica DM1000 microscope with attached Leica DFC295 camera, or a Leica DMRA2 fluorescence microscope (supplementary data).

3.3.4 Micro-computed Tomography (microCT)

Scans of the whole animal were taken at 21 months of age to examine skeletal morphology in detail. A GE eXplore speCZT microCT machine was used to take scans at a resolution of 50 µm/voxel, as before [10]. The mice were assessed for skeletal pathology and general morphological abnormalities using GE Healthcare MicroView software (v2.2) to generate both 2D maximum intensity projection (MIP) images and 3D isosurface renderings [10,22].

3.3.5 Articular Cartilage Thickness and Degeneration

The average thickness of the articular cartilage surfaces of the joints in the knee, elbow, and ankle were taken at 3 locations evenly spaced over the articulating surface being
examined (Fig. 3.1C). Measurements were made from the osteochondral junction to the intact surface of the articular cartilage. Slides were examined at 6 regularly spaced points over 500 µm for the knee in the frontal orientation, and the ankle and elbow in the sagittal orientation [10]. As the talus in the ankle interfaces with at least 3 separate bones in the orientation examined, the individual interfacing surfaces were measured separately (Fig. 3.1C). Blinded examination of the articular cartilage for signs of OA-like degeneration was performed by a single scorer (MP) using the OARSI recommendations for cartilage degeneration scoring in mice [23,24].

3.3.6 CatWalk Gait Analysis

The Noldus CatWalk system (v7.1) was utilized to assess changes in walking gait. Briefly, the animal’s steps are registered as it walks along a glass plate that is imaged by a camera from below and interpreted by the accompanying software as a set of gait parameters [25]. We assessed the rear limb duty cycle (% of time in stand phase of walk cycle) and stride length in age matched 21 month old mice.

3.3.7 Statistical Analysis

All graphical data are presented as the mean ± SEM. Statistical analysis was performed using GraphPad Prism software (v6.0), with two-way ANOVA with Bonferroni’s multiple comparisons test, or t-tests presented as appropriate.

3.4 Results

3.4.1 Mig-6 KO animals are relatively healthy

Female Mig-6 KO mice aged to 21 months appeared to be generally healthy, despite knee and spine pathology discussed below. While male animals were not examined in this experiment, our previous work has shown very similar phenotypes between sexes up to 36 weeks of age [10]. A single KO animal out of 5 died of unknown causes prior to 21 months of age. Mig-6 KO mice aged to 21 months trended towards being slightly heavier than control animals (Supp. Fig. 3.1A).
3.4.2 Calcified nodule formation in 21 month-old Mig-6 KO mice is restricted to knee joint and upper spine

In Mig-6 KO mice aged to 21 months, we observed extensive development of ectopic chondro-osseous nodules primarily in the knee joint, and cervical spine with additional nodules sporadically in the thoracic region of the spine (Fig. 3.2, red arrows). We were unable to locate further calcified nodules in any other joints examined, however non-calcified growths are not detectable using microCT and may be present in areas not histologically examined in detail.

In the cervical spine, calcified tissue was found within the neural canal of the first cervical vertebra (C1, atlas) at the base of the skull of all Mig-6 KO animals examined, but none of the controls (Fig. 3.3A). This calcified tissue is most likely associated with the transverse ligament of the atlas based on its location. Additional calcified nodules were identified surrounding the superior/inferior and transverse costal facets for the ribs in the thoracic spine (Fig. 3.3B). All Mig-6 KO animals demonstrated at least 2 of these nodules within the thoracic spine, but not necessarily at the same vertebral levels. Toluidine blue stained sagittal sections of the spine show masses similar in appearance nodules in the knee joint (below), with pockets of strong GAG stained cartilaginous tissue, bordered by bone-like tissue (Fig. 3.3B). Due to difficulties in sectioning, only a small number of these nodules were captured in histology.

Toluidine blue stained sagittal sections of the knee reveal large, invasive tissue growths in the anterior and posterior region of the joint, composed of large pockets of GAG-rich cartilage-like tissue adjacent to the endogenous articular cartilage surface and integrating with the menisci (Fig. 3.4A, dark blue/purple stain). The tissue extends anteriorly from the meniscus and fills the joint space, follows the patellar ligament, and invades the tibia at the patellar ligament enthesis, disrupting the tibia growth plate (Fig. 3.4A, red asterisk). What appear to be marrow pockets (Fig. 3.4A, red arrows) are surrounded by bone-like, or fibrous tissue. Calcified tissue was examined using microCT, and further confirms that the bone-like tissue in Mig-6 KO animals is calcified, and fully integrates into the underlying bone, effectively immobilizing the joint (Fig. 3.4B). Furthermore, the anterior and posterior cruciate ligaments (ACL/PCL) of Mig-6 KO animals appeared to show cartilage
Figure 3.2 Extensive formation of chondro-osseous nodules in the knees and spine of 21 month old cartilage specific Mig-6 KO mice. Representative 3D isosurface rendering from 50 µm/voxel microCT scans of the whole skeleton of control (top) and KO (bottom) mice. Areas of ectopic nodule development are indicated (red arrowheads) in the knee, and upper spine. Scale bar = 10 mm. N = 3.
**Figure 3.2** Extensive formation of chondro-osseous nodules in the knees and spine of 21 month old cartilage specific Mig-6 KO mice.
Figure 3.3 Ectopic chondro-osseous tissue forms at the base of the skull and the rib joints of 21 month old Mig-6 KO mice. (A) Representative 2D maximum intensity projection (MIP) image of the base of the skull and cervical spine (C1-5) of control (left) and Mig-6 KO mice (right). A calcified mass is present in the region associated with the transverse ligament of the atlas (C1, yellow arrowhead). Scale bar = 2 mm. N=3. (B) Representative toluidine blue stained sagittal section of the thoracic spine shows a costal facet (CF) and rib head (RH) of a control (left) and KO (right) animal. A large chondro-osseous nodule (Nod) is present and integrates the two bones. Scale bar = 200 µm. N = 3
Figure 3.3 Ectopic chondro-osseous tissue forms at the base of the skull and the rib joints of 21 month old Mig-6 KO mice.
Figure 3.4 Ectopic chondro-osseous nodules form in the knee of 21 month old Mig-6 KO mice. (A) Representative toluidine blue stained knee section are shown in the sagittal plane of Mig-6 KO (bottom) and control (top) animals. Mixed tissue chondro-osseous ectopic nodule growths (Nod) are present in the anterior and posterior region of the joint. F = femur, T = tibia. Scale bar = 1 mm. N=3. (B) Representative 2D MIP images of the knee of control (bottom) and KO (top) mice. Scale bar = 1 mm. (C) Representative toluidine blue stained section of the knee joint in the frontal orientation shows cartilage neo-ogenesis (yellow asterisks) in the anterior and posterior cruciate ligaments (ACL/PCL) of Mig-6 KO (bottom), but not control (top) mice. Scale bar = 200 µm. N = 3.
Figure 3.4 Ectopic chondro-osseous nodules form in the knee of 21 month old Mig-6 KO mice
neogenesis along their length, with bone erosion associated with the femoral and tibial ligament entheses (Fig. 3.4C, 3.6A, yellow asterisks), and further localized disruption of the growth plate (Fig. 3.6A, red arrowhead). Nodules were visible upon dissection of 21 month-old Mig-6 KO knee joints as a mass of tissue notably disrupting the patellar ligament (Supp. Fig. 3.2A, red asterisk). Interestingly, 4 week-old Mig-6 KO mice did not show any visible signs of nodule formation in the knee or ankle by histological examination, indicating ectopic tissue is not present at birth (Supp. Fig. 3.3AB).

3.4.3 Ectopic nodules are composed of cells that express Cre

To assess whether ectopic nodules form from Mig-6 KO cells in a cell-autonomous fashion, we utilized the ROSA26<sup>mutTmG</sup> reporter line and 3 imaging techniques to evaluate 5 month-old male age-matched Mig-6 KO ROSA26<sup>mutTmG</sup> reporter mice (n=1 per imaging modality, total n=3). Red fluorescent protein (RFP) is constitutively expressed in all cells until deactivated by Cre recombination, coupled to activation of green fluorescent protein (GFP) expression [19]. Fluorescence imaging using a stereo microscope revealed green fluorescence in the nodule tissue of a disarticulated Mig-6 KO knee joint (Supp. Fig. 3.4A, black asterisk). Similar imaging techniques using progressive whole animal cryosectioning and fluorescence imaging (CryoViz, Bioinvison [26]) showed GFP fluorescence in the ectopic nodules (black asterisks), and articular cartilage of a Mig-6 KO mouse (Supp. Fig. 3.4B). Similarly, histology shows a large proportion of the cells in the ectopic growths adjacent to the meniscus are no longer expressing RFP, and seem to be expressing GFP (Supp. Fig. 3.4C). Taken together, these data indicate that the ectopic growths in the knees of Mig-6 KO animals are derived from cells that have expressed Cre recombinase protein and are likely deficient in Mig-6.

3.4.4 TGFα is not required for ectopic nodule formation in Mig-6 KO mice

TGFα is present in the knee joint and is important during bone and joint development [27]. We mated Tgfa null mice to Mig-6 KO animals to assess the role of TGFα in the development of ectopic nodules in the knee joint. Cartilage-specific Mig-6 KO mice (N=1 male, N=2 female) developed similar ectopic growths in the absence of TGFα at 12 weeks
Figure 3.5 Loss of TGFα does not protect 12 week old cartilage specific Mig-6 KO mice from ectopic tissue formation in the knee. Mice with whole body deletion of Tgfa were bred with Mig-6\textsuperscript{fl/fl}Col2a1-Cre mice to create double KO animals. Representative toluidine blue stained sagittal knee sections show similar nodule formation (yellow asterisk) and growth plate disruption (red arrows) in Tgfa null Mig-6 KO mice and control Tgfa wild type Mig-6 KO mice. Cre negative controls did not develop ectopic tissue. F = femur, T = tibia. Scale bar = 200 µm. N = 1 male, 2 female.
Figure 3.5 Loss of TGFα does not protect 12 week old cartilage specific Mig-6 KO mice from ectopic tissue formation in the knee
of age (Fig. 3.5, yellow asterisks), suggesting that TGFα is not required for the formation of these growths.

3.4.5 Thicker articular cartilage is present in multiple joints of 21 month old Mig-6 KO

The role of Mig-6 in cartilage anabolism in 21 month old mice was examined by measuring the thickness of the articular cartilage from the subchondral bone to the intact cartilage surface of the knee, ankle, and elbow joints (Fig. 3.1C).

Frontally oriented toluidine blue stained sections of the knee show slightly weaker GAG staining in the expanded non-calcified articular cartilage relative to control animals (Fig. 3.6A). The surface of the articular cartilage in KO animals does not show extensive fibrillation, and is relatively smooth and continuous (Fig. 3.6A, inset). However, the contour of the joint in KO shows unusual hills and valleys typically in areas adjacent to cartilage-like masses in the ligaments or meniscus that may impinge on the articular cartilage surface (Fig. 3.6A, dashed red line LFC). The articular cartilage thickness was increased in the lateral femoral condyle (LFC) and medial femoral condyle (MFC) of KO animals, with a further trend towards an increase in the lateral tibial plateau (LTP) (Fig. 3.6B). However, the medial tibial plateau (MTP) showed no large differences (Fig. 3.6B).

To evaluate cartilage degeneration within the knee, 6 sections spanning at least 500 µm were evaluated and degeneration scores summed across the depth of the quadrant based on OARSI cartilage degeneration scoring guidelines [23,24]. OARSI scoring demonstrated a non-significant increase in cartilage degeneration in KO animals in the LTP, MFC, and MTP with a clear increase in degeneration observed in the LFC relative to controls (Fig. 3.6C). The total joint score (sum of LFC/LTP/MFC/MTP) was significantly higher in the KO animals compared to controls (Fig. 3.6C). The majority of damage observed was related to localized loss of GAG staining, with little disruption of the underlying cartilage structure with the exception of one KO animal that showed signs of matrix loss (Fig 3.5C).
Figure 3.6 Articular cartilage thickness is enhanced in the knee joints of 21 month old cartilage specific Mig-6 KO mice. (A) Representative images of toluidine blue stained frontally sectioned knees of control (left) and Mig-6 KO (right) mice. A magnified view of the articular cartilage surface shows a relatively smooth surface in KO mice (Inset). Cartilage neogenesis (yellow asterisk) in the ACL/PCL of KO animals. The joint contour is disrupted in KO mice (red dashed line). Local disruption of the growth plate (red arrowhead) is also present. LFC = lateral femoral condyle, LTP = lateral tibial plateau, MFC = medial femoral condyle, MTP = medial tibial plateau. Scale bars = 200 µm. N=3. (B) Measured articular cartilage thickness from subchondral bone to intact cartilage surface, see Fig. 3.1, for details on measurements. Articular cartilage thickness is enhanced in the LFC and MFC of Mig-6 KO mice. (C) Articular cartilage degeneration scores based on OARSI recommendations [23]. Mig-6 KO mice show increased cartilage degeneration in the LFC and Total Joint. The majority of scores consisted of GAG depletion without matrix breakdown. (A-C) Individual data points presented with mean ± SEM. Data analyzed by two-way ANOVA with Bonferroni’s multiple comparisons test, * p < 0.05, *** p < 0.005, **** p < 0.001. N = 3.
Figure 3.6 Articular cartilage thickness is enhanced in the knee joints of 21 month old cartilage specific Mig-6 KO mice
The gait patterns of 21 month old control and Mig-6 KO mice (N=4, age matched) were examined immediately prior to sacrifice. The duty cycle (% time in stand phase of walk cycle) of the hind limbs showed a non-significant decrease in KO animals relative to controls (Supp. Fig. 3.5A). No statistically significant differences were noted in the rear limb stride length of Mig-6 KO mice (Supp. Fig. 3.5B).

The ankle was then examined to evaluate a joint that does not show any apparent formation of chondro-osseous nodules, which may influence degeneration through increased joint imbalance and abnormal loading [2,28]. We evaluated a number of joint interfaces (notation: Measured surface –opposite interfacing surface, ex. Tibia-talus), as some bones have multiple joint surfaces (Fig. 3.1C). The joint surfaces of the talus (Tal), navicular (Nav) and calcaneus (Calc) all showed increased articular cartilage thickness (30-100%) in KO animals, in contrast to the tibia (Tib) (Fig. 3.7AC). The GAG staining in the non-calcified articular cartilage also appeared more intense in Mig-6 KO mice, indicating there may be increased proteoglycan content (Fig. 3.7A). No evidence for degeneration was seen in ankle joints of any animal examined. Similar findings are seen in the ankle of 4 week old Mig-6 KO mice, with statistically significant increases in the talus-navicular joint, and non-significant increases the talus-calcaneus, and talus portion of the tibia-talus joint (Supp. Fig. 3.3BC). Intermediate increased articular cartilage thickness in 4 week old Mig-6 KO mice may indicate increased cartilage expansion relative to their control littermates as the animals grow.

The elbow joint also appears unaffected by nodules in KO animals based on microCT analysis (Fig. 3.2) and histology (Fig. 3.7B). Measurements of the radius, ulna, and humerus in KO animals show an increase (≥100%) in the thickness of the articular cartilage relative to control animals (Fig. 3.7D). The thickened articular cartilage in KO animals stained robustly for GAG, particularly when compared to control animals (Fig. 3.7B). Interestingly, in each Mig-6 KO animal we also observed what may be cartilage neogenesis extending proximally from the distal humerus (trochlea of the humerus) into the olecranon fossa and coronoid/radial fossa (Fig. 3.7B, black arrowheads).
Figure 3.7 Articular cartilage thickness is enhanced in the ankle, and elbow of 21 month old cartilage specific Mig-6 KO mice. (A) Representative toluidine blue stained sagittal ankle sections of control (left) and Mig-6 KO (right) mice. T = tibia, Ta = talus, N = navicular, C = calcaneus. (B) Representative toluidine blue stained sections of sagittal elbows of control (left) and Mig-6 KO (right) mice. Evidence of cartilage neogenesis (black arrows). H = humerus, R = radius, U = ulna. (C) Ankle articular cartilage thickness was increased in Mig-6 KO mice in all joint interfaces examined excluding the tibia of the tibiotalar joint (Tib-tal). (D) Elbow articular cartilage thickness was significantly increased in Mig-6 KO mice in all joint surfaces examined. (A-C) Individual data points presented with mean ± SEM. Data analyzed by two-way ANOVA with Bonferroni’s multiple comparisons test, * p < 0.05, ** p < 0.01, *** p < 0.005, **** p < 0.001. All scale bars = 200 µm. N = 3.
**Figure 3.7** Articular cartilage thickness is enhanced in the ankle, and elbow of 21 month old cartilage specific Mig-6 KO mice
3.4.6 Post-natal induction of Mig-6 deletion targets a subpopulation of articular chondrocytes

To evaluate the role of Mig-6 in postnatal cartilage, we induced Cre activity at 3 weeks of age using mice with tamoxifen inducible Col2a1-CreER and conditional deletion Mig-6<sup>fl/fl</sup> alleles [16,21]. Mice positive and negative for CreER received either tamoxifen or vehicle in 5 injections over 5 days to adequately induce recombination (Fig. 3.1B). We assessed tamoxifen-induced, Cre-mediated deletion of Mig-6 by examining PCR products for recombination (or the intact allele) with two primers flanking the ‘floxed’ region and one within. Only the ear and xyphoid cartilage of CreER(+)Mig-6<sup>fl/fl</sup> animals injected with tamoxifen showed the presence of the recombined (deleted) Mig-6 allele (Supp. Fig. 3.6A). The intact band is still present in these animals, indicating either that the recombination efficiency is not 100% or contaminating tissue was present. A single additional trial was conducted with animals bearing the ROSA26 targeted mTmG reporter discussed above [19]. Immunostaining of sections of the knee joint indicate the presence of GFP expression in chondrocytes in at least 2 regions of the tibia of a CreER(+)Mig-6<sup>fl/fl</sup> tamoxifen injected animal, but not in controls (Supp. Fig. 3.6B). This would indicate that at least a subpopulation of chondrocytes is undergoing recombination, however the regional variability within the same joint of the animal examined does raise concerns regarding recombination efficiency. Additional trials need to be performed to confirm adequate deletion of Mig-6 in these animals.

However, induction of Cre activity in CreER mice with tamoxifen at 3 weeks and aging to 12 weeks did not seem to cause any general health concerns. Male CreER mice did not show any trends or statistically significant differences in weight after tamoxifen injection (Supp. Fig. 3.1B). Female animals were not examined in tamoxifen induced CreER experiments to reduce potential systemic effects of tamoxifen and female hormones.

3.4.7 Post-natal induction of Mig-6 deletion increases lateral tibia articular cartilage thickness

We examined the thickness of knee articular cartilage thickness as described above (Fig. 3.1C). Only the LTP quadrant of the knee showed a statistically significant increase in cartilage thickness for tamoxifen injected CreER(+) mice relative to vehicle, but not the
tamoxifen injected CreER(-) controls (Fig. 3.8AB). No observable effects on the growth plate, menisci, or anterior/posterior cruciate ligaments were noted (Fig. 3.8A). Furthermore, animals did not demonstrate any evidence of nodule formation in the knee during a gross evaluation of knee morphology during dissection (Supp. Fig. 3.2B). The number of chondrocytes in a defined region (200 µm x 100 µm box) of the MTP was examined to evaluate chondrocyte proliferation following post-natal Mig-6 deletion. No statistically significant differences were observed in tamoxifen injected CreER(+) animals when compared to any control groups (Fig. 3.8C).

Following the knee, we examined the elbow joint of CreER animals, since 21 month-old KO mice showed the largest increase in cartilage thickness in this joint. The elbow of tamoxifen injected CreER(+) mice did not show thicker articular cartilage in the humerus, ulna, or radius when compared to control groups (Fig. 3.9AB). Furthermore, GAG staining appeared similar between groups (Fig. 3.9A).

3.5 Discussion

In this study we demonstrated that at 21 months of age, mice with cartilage-specific deletion of Mig-6 develop partially calcified chondro-osseous nodules in the knee and spine, and furthermore show increased articular cartilage thickness with a minor increase in cartilage degeneration in the knee. We also demonstrated that post-natal deletion of Mig-6 at 3 weeks of age was insufficient to induce similar cartilage anabolism when examined 9 weeks later in mice 12 weeks of age.

These findings complement previous studies that examined Mig-6 and EGFR in mouse joints using various models. Whole body deletion of Mig-6 first demonstrated that Mig-6 plays an important role in homeostasis of joint tissues, with animals rapidly developing debilitating joint nodules in the knee, ankle and temporo-mandibular joint (TMJ) and cartilage degeneration in the knee [16,17]. The majority of these animals died by 3 months of age due to difficulty feeding with TMJ impairment. This was resolved in a study that used a Prx-1 driven Cre to delete Mig-6 in the limb mesenchyme and resultant whole joint, first demonstrating a transient anabolic effect in the knee that gave way to cartilage degeneration by 16 weeks of age [12]. Use of two different but functionally similar Col2a1-
Figure 3.8. Post-natal deletion of Mig-6 at 3 weeks mildly increased articular cartilage thickness in the knee of 12 week old mice. (A) Representative images of toluidine blue stained frontal knee sections of tamoxifen (bottom) and vehicle (corn oil, top) injected, Col2-CreER(-) [left] and Col2-CreER(+) [right] 12 week old mice. LFC = lateral femoral condyle, LTP = lateral tibial plateau, MFC = medial femoral condyle, MTP = medial tibial plateau. (B) Articular cartilage thickness was increased only in the LTP of tamoxifen injected CreER(+) mice relative to vehicle controls. (C) Number of chondrocytes in the articular cartilage of the MTP did not increase in tamoxifen injected CreER(+) mice or controls. (A-C) Individual data point presented with mean ± SEM. Data analyzed by two-way or one-way ANOVA with Bonferroni’s multiple comparisons test. **p < 0.01. Scale bar = 200 µm. N ≥ 4.
Figure 3.8 Post-natal deletion of Mig-6 at 3 weeks mildly increased articular cartilage thickness in the knee of 12 week old mice.
Figure 3.9. Post-natal deletion of *Mig-6* at 3 weeks did not increase the articular cartilage thickness in the elbow of 12 week old mice. (A) Representative toluidine blue stained sagittal elbow sections of tamoxifen (bottom) and vehicle (corn oil, top) injected, Col2-CreER(-) [left] and Col2-CreER(+) [right] 12 week old mice. H = humerus, R = radius, U = ulna. Scale bar = 200 µm. (B) Articular cartilage thickness was not increased for any joint surface examined. Individual data point presented with mean ± SEM. N ≥ 4. Data analyzed by two-way ANOVA with Bonferroni’s multiple comparisons test.
Figure 3.9 Post-natal deletion of Mig-6 at 3 weeks did not increase the articular cartilage thickness in the elbow of 12 week old mice.
Cre driver mice further restricted Mig-6 deletion to cartilaginous tissues, and demonstrated that the thickened articular cartilage in Mig-6 KO mice persisted until 36 weeks and 15 months of age, although these animals still developed ectopic growths in the knee joint [10,11].

The role of EGFR signalling and Mig-6 in human musculoskeletal diseases is not entirely clear. However, mutations in EGFR and Mig-6 have been repeatedly implicated in lung cancer [29,30] and EGFR signalling may be involved in the formation of various types of sarcomas [31,32]. The phenotype we have described here, and previously [10], resembles the pathology associated with chondromatosis and exostosis in which cartilaginous extensions form from the bone or joint, and progressively ossify [33–35]. Studies have shown that Ptpn11 (SHP2), which facilitates EGFR signaling, is involved in the regulation growth of cartilage and bone and that loss of SHP2 results in metachondromatosis in the ankle and knee [36–38]. However in these studies, loss of SHP2 generally decreased downstream signalling to MEK/ERK, which is converse to the effects of loss of Mig-6, as previously observed [10–13,16]. Considering this, it is possible that the Mig-6 KO phenotypes observed are not entirely due to EGFR modulation and may be partially mediated by interactions with other receptor tyrosine kinase proteins like MET, protein-protein interactions with c-Abl, or others [15,39]. On the other hand, Exostosin 1 and 2 (EXT1/2) are important in the synthesis of heparan sulfate (HS) GAG chains, and partial or complete loss of Ext1 has been associated with both hereditary and spontaneous osteochondromas, similar in structure to the nodules we have described [35,40,41]. Heparan sulfate binds the EGF ligand heparin-binding egf-like protein (HB-EGF), and loss of ECM binding of HB-EGF or similar EGF-like ligand amphiregulin may be related to the chondroma phenotypes observed in Ext1 KO and Mig-6 KO mice [10,41,42]. Calcified tissue was also identified in the neural canal of the C1 vertebra (atlas), and seemed to be associated with the transverse ligament based on the anatomical location in microCT scans. Osteochondromas have been previously described in this same area, further indicating that there may be human pathologies similar to the phenotypes we observe here [43–45]. Additional research is needed to solidify the role of EGFR signalling, and regulation of this pathway by Mig-6 and others in the formation of osteochondromas and related diseases.
Interestingly, the nodule formation described here appears to be independent of TGFα, as shown by analyses of double KO mice that formed similar nodules as cartilage-specific Mig-6 KO mice. Although our data show that TGFα is a major EGFR ligand in the joint [5–7,27,46,47], this could be due to a number of reasons. For example, other EGFR ligands or different receptor tyrosine kinases could compensate for reduced TGFα signaling [30,48]. Alternatively, the nodule formation might be independent of an upstream receptor tyrosine kinase [49]. These possibilities will be investigated in the future. Additionally, while a male and two female trials were grouped together for analysis, we have consistently observed similar pathology between sexes in Mig-6 KO experiments ([10] and unpublished), and do not believe this has introduced any error.

To our knowledge, this is the first study to demonstrate that deletion of Mig-6 in mice can enhance the anabolic characteristics of articular cartilage that persist through the lifetime of the animal in multiple joints beyond the knee. We had previously shown that articular cartilage thickness in cartilage-specific Mig-6 KO animals was increased up to 36 weeks of age, and others had shown that this phenotype may persist up to 15 months [10,11], but these studies were restricted to the knee joints that also showed disruptive nodule formation. At 21 months of age, the articular cartilage of the elbow and ankle showed robustly increased articular cartilage thickness and staining for GAGs. The articular cartilage of the knee showed increased thickness in some quadrants of the joint, with the caveat of increased cartilage degeneration in the form of reduced GAG staining. It is important to note that despite GAG loss, the structure of the cartilage seemed to be largely spared in the KO animals examined. Early OA is often preceded by a loss of GAG content that is followed by collagen II loss and matrix destruction [2], therefore future studies may need to prolong the time point examined to fully evaluate OA development in these animals. That being said, mice often show mild-moderate OA by this time in excess of what we have observed in this study [28]. While we observed minimal effects to the gait of our Mig-6 KO mice, it may be safe to speculate that normal physiological loading and articulation in the immobilized knee joints of Mig-6 KO is highly disrupted by nodule formation. Indeed, destabilization of joint loading and alignment forms the basis of many of the rodent models we currently employ to study OA development [28]. Therefore, it is
remarkable to us that more extensive disruption of the articular cartilage was not observed in these animals.

We do not yet fully understand why our animals do not show more detectable changes to gait using the CatWalk system. Nodule formation in Mig-6 KO mice causes severe dysfunction in the articulation of the knee joint, and prevents normal extension [10,11]. However, due to some heterogeneity in the degree and location of nodule formation in the knees of Mig-6 KO mice [10], the signal may be variable enough to have been lost in the experimental noise. Furthermore, affected animals may adapt with altered hip and ankle use to mask the effects of knee dysfunction. Future studies may need additional imaging techniques to examine joint function in Mig-6 KO animals in real time [50].

In this study we also postnatally deleted Mig-6 in 3 week old mice by tamoxifen induction of Col2a1-CreER and examined the animals at 12 weeks of age. Tamoxifen induced CreER(+) animals did not appear to develop any evidence of chondro-osseous nodules, however these formations may have been too small to detect using the techniques we employed at that time point. Despite previous results indicating that enhanced cartilage thickness is present by 12 weeks of age in cartilage specific Mig-6 KO mice [10], and perhaps at 4 weeks of age, we observed very minor effects on cartilage anabolism in this study. In fact, we have also shown that nodule development may initiate as early as 6 weeks of age in the knee [10]. This may indicate that Mig-6 is more important during the development of the joint, perhaps by enhancing growth of the cartilage structure during embryonic or neo-natal growth, or through the modulation of resident chondrocyte/osteoblast precursor cells [13]. However, in depth examination of developmental time points has not yet been conducted. Future studies may need to examine additional time points later in the life mice with post-natal deletion of Mig-6, and embryonic/neonatal time points of constitutive cartilage specific Mig-6 KO mice.

There are limitations to the methodology used in this study. Early evidence using the ROSA26°TmG based reporter [19] indicates that only a subpopulation of articular chondrocytes may be undergoing CreER-mediated recombination. Data produced in our lab indicate that heterozygous deletion of Mig-6 is insufficient to generate the articular
cartilage, or ectopic nodule phenotype observed in homozygous Mig-6 KO mice ([10,11] and unpublished). Compensation for a single Mig-6 copy by similar negative regulators of EGFR signalling, or partial compensation by other means may overcome the mechanisms driving the pathology observed with homozygous deletion [10,51,52]. This may be further complicated by the fact that relative to developing mice, mature animals more infrequently express the Col2a1 gene which drives CreER expression in our model [21,53]. While at the age of 3 weeks our animals should have been actively producing some collagen II, it is possible that recombination efficiency would be better using an alternative CreER driver line [54]. This is of critical importance considering that our Col2a1-Cre (non-inducible) Mig-6 KO mice show recombination in cartilaginous tissues including the meniscus and ligaments [10,55], however we were not able to show recombination in tissues other than the articular cartilage in our post-natally induced Mig-6 KO mice. Whole body Mig-6 KO animals [12,16,17] consistently demonstrate more severe chondro-osseous nodule development than chondrocyte specific models [10,11] potentially due to the involvement of additional joint tissue involved.

In conclusion, our data suggest that EGFR signalling and modulation by Mig-6 in synovial joint tissues is highly complex, and effects seem to be both time- and cell-specific. However, induction of thicker articular cartilage in response to loss of Mig-6 may be promising for future treatment of OA and other joint degenerative diseases. Further research is required to evaluate these pathways in joint health and disease.

3.6 Acknowledgements

M.A.P. was supported by a Canadian Institute of Health Research (CIHR) doctoral scholarship and in part by the Joint Motion Program a CIHR Training Program in Musculoskeletal Health Research and Leadership. F.B holds the Canada Research Chair in Musculoskeletal Research. This work was supported by CIHR Operating Grant MOP 86574 to F.B.
3.7 Supplementary Figures

Supplementary Figure 3.1 Weights of 21 month old Mig-6 KO and 12 week old postnatally induced Mig-6 KO are comparable to controls. (A) Weights of 21 month old female cartilage-specific Mig-6 KO mice and controls taken immediately prior to sacrifice. Mig-6 KO mice trend towards an increased weight, however the difference is only 2.500 ± 1.026 g and not significant. (B) Weights taken immediately before sacrifice of CreER(-) and CreER(+) injected with tamoxifen or vehicle (corn oil) at 3 weeks of age, and followed until 12 weeks. (A-B) Individual data points presented with mean ± SEM. N ≥ 4. Data analyzed with t-test, or one-way ANOVA with Bonferroni’s multiple comparisions test.
Supplementary Figure 3.1 Weights of 21 month old Mig-6 KO and 12 week old postnatally induced Mig-6 KO are comparable to controls.
Supplementary Figure 3.2 Ectopic nodules are evident on gross examination of 21 month old Mig-6 KO, but not postnatally induced Mig-6 KO mice. (A) Representative images of gross examination of the skinned legs of 21 month old Mig-6 KO mice show ectopic tissue (red asterisk) distending out from the patellar ligament (black asterisk) of the knee joint. F = femur. T = tibia. (B) Representative images of gross examination of the knees of 12 week old postnatally induced Mig-6 KO mice show no abnormalities. (A-B) Scale bars = 200 µm. N ≥ 3.
Supplementary Figure 3.2 Ectopic nodules are evident on gross examination of 21 month old Mig-6 KO, but not postnatally induced Mig-6 KO mice.
Supplementary Figure 3.3 Increased articular cartilage thickness in the ankle, and absence of ectopic tissue in the ankle and knee of 4 week old Mig-6 KO mice. (A) Representative images of toluidine blue stained sagittal knee sections of 4 week old control (left) and Mig-6 KO (right) mice. T = tibia, F = femur. Scale bar = 200 µm. N = 6. (B) Representative images of toluidine blue stained sagittal ankle sections of 4 week old control (left) and Mig-6 KO (right) mice. T = tibia, Ta = talus, N = navicular, C = calcaneus. Scale bar = 200 µm. N = 3. (C) Articular cartilage thickness in the ankle is increased in the talus-navicular joint of 4 week old Mig-6 KO mice. Individual data points are presented with mean ± SEM. p < 0.05, p < 0.005. N = 3.
Supplementary Figure 3.3 Increased articular cartilage thickness in the ankle, and absence of ectopic tissue in the ankle and knee of 4 week old Mig-6 KO mice
Supplementary Figure 3.4. Chondro-osseous nodules of 5 month old Mig-6 KO mice are composed of cells that expressed Cre. (A) The ROSA26\textsuperscript{mTmG} reporter was utilized with Mig-6\textsuperscript{fl/fl}Col2a1-Cre mice to visualize cells that have expressed Cre at some point and activated GFP expression (deactivating RFP expression). Representative image of the disarticulated knee joint (femur removed) of a 5 month old Mig-6\textsuperscript{fl/fl}Col2a1Cre\textsuperscript{+/−} ROSA26\textsuperscript{mTmG} mouse shows GFP fluorescence in the nodule (black asterisk) that forms in the anterior joint space. Cartilage in the patella, tibia, fibula and cruciate ligaments is also positive for GFP. T = tibia, P = patella. Scale bar = 1 mm. N = 1. (B) Representative fluorescence images of whole animal cryosectioning shows GFP signal in the knee cartilage of 5 month old Mig-6 WT and Mig-6 KO mice positive for both Cre and the mTmG reporter. GFP is also visible in the ectopic tissue (black asterisks) that forms in the knee joint of Mig-6 KO. F = femur, T = tibia, P = patella. N = 1. Scale bar = 1 mm. (C) Fluorescence imaging of sagittal knee sections of a Mig-6 KO mouse positive for both Cre and the mTmG reporter show RFP negative/GFP positive cells in the ectopic nodule (Nod, dashed white outline) in the anterior knee joint. F = femur, T = tibia, M = meniscus. Scale bar = 100 µm. N = 1.
Supplementary Figure 3.4. Chondro-osseous nodules of 5 month old Mig-6 KO mice are composed of cells that expressed Cre
Supplementary Figure 3.5 Gait in 21 month old *Mig-6* KO mice. The gait of age matched mice was examined using a Noldus CatWalk apparatus and software (v7.1) for changes possibly indicative of joint malfunction and/or pain-like behaviours. (A) The duty cycle (% of time spent in the stand phase of the walk cycle) of the rear limbs trended towards a decrease in *Mig-6* KO mice but was not statistically significant (p=0.073) (B) Stride length of the rear limbs of *Mig-6* KO mice was similar to control mice. (A-B) Individual data points presented with mean ± SEM. N = 4 age matched. Data analyzed by t-test.
Supplementary Figure 3.5 Gait in 21 month old Mig-6 KO mice.
Supplementary Figure 3.6 Evidence of Cre mediated recombination in 12 week old tamoxifen induced Mig-6 Col2-CreER mice. (A) Primers flanking the ‘floxed’ region and one within the Mig-6 allele were used to assess Mig-6 recombination and resultant excision. Only the ear and xyphoid tissues of the tamoxifen injected CreER(+) mice were strongly positive for the product of recombination (top band). (B) An additional trial was bred with ROSA<sup>mtmG</sup> reporter mice to show localization of Cre recombination. Expression of GFP follows recombination that was probed for using immunostaining of tamoxifen or vehicle injected CreER(+) knee joints. GFP positive cells (brown staining) are present in the tibia tissue of tamoxifen injected CreER(+) mice (two locations shown #1, and #2, right panels). Vehicle injected controls and no primary exposed control tissue did not show evidence of GFP staining. Methyl green was used as a counter-stain. Scale bar = 100 µm.
Supplementary Figure 3.6 Evidence of Cre mediated recombination in 12 week old tamoxifen induced Mig-6 Col2-CreER mice
3.8 References


Chapter 4

4 Deletion Of Dual Specificity Phosphatase 1 Does Not Predispose Mice To Increased Spontaneous Osteoarthritis

These data have been submitted to PLOS ONE 2015-07-30, and are awaiting review:

Pest MA, Pest CA, Bellini MR, Feng Q, Beier F. Deletion of Dual Specificity Phosphatase 1 Does Not Predispose Mice to Increased Spontaneous Osteoarthritis. PLOS ONE, Submitted 2015-07-30

4.1 Abstract

**Background:** Osteoarthritis (OA) is a degenerative joint disease with poorly understood etiology and pathobiology. Mitogen activated protein kinases (MAPKs) including ERK and p38 play important roles in the mediation of downstream pathways involved in cartilage degenerative processes. Dual specificity phosphatase 1 (DUSP-1) dephosphorylates the threonine/serine and tyrosine sites on ERK and p38, causing deactivation of downstream signalling. In this study we examined the role of DUSP-1 in spontaneous OA development at 21 months of age using a genetically modified mouse model deficient in *Dusp1* (DUSP-1 knockout mouse).

**Results:** Utilizing histochemical stains of paraffin embedded knee joint sections in *Dusp1* knockout and wild type female and male mice, we showed similar structural progression of cartilage degeneration associated with OA at 21 months of age. A semi-quantitative cartilage degeneration scoring system also demonstrated similar scores in the various aspects of the knee joint articular cartilage in *Dusp1* knockout and control mice. Examination of overall articular cartilage thickness in the knee joint further showed similar results between *Dusp1* knockout and wild type mice. Immunostaining for cartilage neoepitopes DIPEN, TEGE and C1,2C was similar in the cartilage lesion sites and chondrocyte pericellular matrix of both experimental groups. Likewise, immunostaining for phosphoERK and MMP-13 showed similar intensity and localization between groups. SOX9 immunostaining demonstrated a trend towards a decreased number of positive cells
in *Dusp1* knockout mice, with correspondingly decreased staining intensity. Analysis of animal walking patterns (gait) did not show a discernable difference between groups.

**Conclusion:** Loss of DUSP-1 does not cause changes in cartilage degeneration and gait in a mouse model of spontaneous OA at 21 months of age. Subtle changes were observed in SOX9 immunostaining that may prove promising for future studies examining the role of DUSPs in cartilage and OA, as well as models of post-traumatic OA.

### 4.2 Introduction

Osteoarthritis (OA) is a degenerative joint disease that is estimated to afflict at least 10% of the US population over the age of 25 [1]. Symptoms of OA include joint pain and stiffness that can become severe enough to limit activity and ability to work. This results in a substantial loss for both patient quality of life and the economy through missed work hours and direct healthcare costs [2]. Currently, pharmacological interventions only mitigate the symptoms of the disease and do not slow, stop or reverse the underlying joint damage associated with OA, and so further research is needed [2]. While the etiology and pathophysiology of OA is poorly understood, research has shown that contrary to previous beliefs that OA was primarily a disease of ‘wear and tear’, there is a complex set of cellular changes linked to genetic factors and altered biomechanics that occurs in the joint tissues and influences disease initiation and progression [3].

At the tissue level, chondrocytes are the only active cellular component of the cartilage that caps the bone in articular joints such as the knee, elbow and ankle. These cells maintain tissue homeostasis by balancing anabolic buildup and catabolic turnover of surrounding extracellular matrix (ECM) proteins [2]. The ECM forms the vast majority of cartilage tissue and consists of a complex network of mostly collagen II and sulfated glycosaminoglycan (GAG) containing proteoglycans like aggrecan [2]. Matrix production is largely controlled by the transcription factor SRY (sex determining region Y)-box 9 (SOX9), which also acts as the master regulator of the chondrocyte phenotype [2,4]. Conversely, matrix is catabolized by a number of proteinases produced by chondrocytes including matrix metalloproteinase (MMP) -3 and MMP-13, as well as various
aggrecanases [2]. An imbalance in ECM turnover and tissue homeostasis is thought to be one of the primary underlying reasons for cartilage degeneration in OA. This imbalance is largely driven by certain growth factors and inflammatory cytokine signalling [2].

Various growth factors have been implicated in OA, including transforming growth factor beta (TGFβ), fibroblast growth factors (FGF), and transforming growth factor alpha (TGFα) [5–7]. Previous studies in our lab have shown TGFα mediated activation of epidermal growth factor receptor (EGFR) induces cartilage degeneration in vitro, and inhibition of EGFR in vivo partially protects against surgically induced osteoarthritis in rats [8,9]. A portion of the cellular response to TGFα and other EGFR ligands is mediated by activation of downstream mitogen activated protein kinases (MAPKs) such as extracellular signal-regulated kinase 1 and 2 (ERK1/2) and p38 MAPK [8,10–12]. ERK activation has been shown to induce downstream targets involved in OA including MMPs, and blockade of MAPK kinase (MEK)/ERK signalling attenuated cartilage degeneration using in vitro cartilage explant models, and in vivo rabbit models of OA [8,12,13]. Inhibition of p38 has also been shown to partially attenuate production of catabolic MMPs, and reduce cartilage degeneration and pain-like behaviours in a rat monoiodoacetate (MIA) induced OA model [14,15]. Furthermore, p38 mediates part of the activity of various inflammatory cytokines including IL-1β and TNFα, which are important in both inflammatory arthritis and osteoarthritis [2,16].

Dual-specificity phosphatase 1 (DUSP-1), also known as MAPK phosphatase-1 (MKP-1), and other DUSP proteins are negative regulators of MAPK signaling [17]. With the ability to dephosphorylate phosphoserine/phosphothreonine and phosphotyrosine sites, DUSP-1 can deactivate phosphorylated ERK and p38 [17,18]. DUSP-1 has been shown to attenuate MAPK signaling, effectively reducing osteolysis and cartilage degeneration in studies utilizing mouse models of lipopolysaccharide (LPS) induced inflammatory bone loss and collagen induced arthritis (CIA) [16,19,20]. Inhibition of DUSP-1 also increased chondrocyte apoptosis, which was exacerbated by the cytokine TNFα in cell culture [21]. Our own studies have shown induction of Dusp1 expression in chondrocytes by pharmacological inhibition of phosphatidylinositol-3-kinase (PI3K) signaling [22].
However, the role of DUSP-1 in OA has not been closely examined using in vivo animal models.

In this study, we investigated the role of DUSP-1 in spontaneously occurring OA by aging mice genetically deficient in Dusp1. We demonstrate that both female and male mutant animals show similar disease progression to controls at 21 months of age.

4.3 Methods

4.3.1 Animals

All animals were bred in-house, raised, and sacrificed in accordance to the ethical guidelines of the Canadian Council on Animal Care (CCAC). Animal use protocols were approved by the Council on Animal Care at Western University – Canada (Animal Use Permit: 2007-045). Mice were housed on a 12 hour light/dark cycle in standard shoebox caging with free access to mouse chow and water, but without access to running wheels or other exercise based enrichments. Mice with a whole body deletion for Dusp1 were obtained from QF on a mixed C57Bl/6 and 129S2 background [23,24]. Trials were setup in cages containing at least 1 pair of littermate matched wild type and knockout animals, with an average of 3-4 mice per cage for the duration of the experiment. All animals were weighed prior to euthanization by asphyxiation with CO2. Wild type (Dusp1+\(^+\), WT), heterozygous (Dusp1\(^{+/−}\), Het), and Dusp1 knockout (Dusp1\(^{-/−}\), KO) mice were genotyped using standard PCR techniques on ear tissue biopsies using the following primers: Dusp1 For1 - 5-CCA GGT ACT GTG TCG GTG GTG C-3, Dusp1 For2 - 5-TGC CTG CTC TTT ACT GAA GGC TC-3, Dusp1 Rev1 - 5-CCT GGC ACA ATC CTC CTA GAC-3 [25].

4.3.2 Histology

Limbs were collected and fixed in 4% paraformaldehyde for 24 hours. The intact joints were then decalcified in 5% ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS, pH 7.0) for 10 to 12 days at room temperature. Knee joints were processed and embedded in paraffin to obtain sections 5 μm thick in the frontal orientation. Staining
with toluidine blue for loss of glycosaminoglycans (GAGs) and cartilage structural changes was conducted as previously described [26].

4.3.3 Articular Cartilage Evaluation

To evaluate OA related joint degeneration and articular cartilage thickness, 5 evenly spaced sections spanning a region approximately 500 µm were examined. OA related damage was assessed using the OARSI approved histological scoring system for mice [27], with all four quadrants of the joint (medial/lateral tibia and femur) scored by at least 2 blinded observers. As previously described, the average articular cartilage thickness was measured from the subchondral bone to the surface of the intact articular cartilage at three evenly spaced points along the width of each knee joint quadrant (Fig. 4.2C for illustration) using frontally oriented sections [26].

4.3.4 Immunohistochemistry

Primary antibodies for cartilage and bone matrix breakdown products were received as a gift from John Mort and included anti-TEGE (aggrecanase mediated aggrecan cleavage neoepitope), anti-DIPEN (matrix metalloproteinase mediated aggrecan cleavage neoepitope) and anti-C1,2C (a collagen I and II cleavage neoepitope) and were utilized as previously described [28–31]. Additional antibodies were obtained from their various manufacturers: MMP-13 (Proteintech), SOX9 (R&D Systems), phosphoERK1/2 (Cell Signalling). Sections were incubated for 15 minutes in 3% H$_2$O$_2$ in methanol to eliminate endogenous peroxidase activity, followed by blocking in 5% goat or donkey serum in PBS, and overnight incubation with primary antibody at 4ºC. Sections were incubated with the appropriate secondary antibody (goat anti-rabbit, or donkey anti-goat, Santa Cruz) conjugated to horseradish peroxidase (HRP) and developed using DAB+ chromogen (Dako Canada). Both antibody isotype and ‘no primary antibody’ controls were also performed (data not shown). Sections were counterstained with methyl green, dehydrated in solutions of increasing ethanol concentration ending in xylene, and cover slipped.
4.3.5 CatWalk Gait Analysis

Changes in gait and weight bearing were assessed using the Noldus CatWalk system [32–34]. Animals were transferred to a behavioral facility 2 days prior to assessment. In brief, animals were allowed to walk freely through a semi-enclosed tunnel along an internally illuminated glass plate. In a dark room the scattering of the light caused by interaction with the animal’s paws is detected by a camera, and subsequently interpreted by the accompanying software (Noldus CatWalk v7.1) as various gait parameters. Stride length is calculated as the average distance between paw strikes for each limb. Duty cycle is an expression of the percentage of time the paw remains in contact with the glass plate, relative to the total gate cycle as shown in this equation: Duty Cycle = \((\text{Stand Time}/(\text{Stand Time} + \text{Swing Time}))\) x 100%. Print area and paw intensity are related to the amount of weight the animal bears on an individual paw, with the maximal contact area and maximal intensity of light measured respectively.

4.3.6 Statistical Analysis

All data were analyzed for statistical significance using GraphPad Prism (v6.0) and the appropriate statistical techniques (two-way ANOVA or t-tests as indicated). Outliers were assessed using the Grubbs’ outlier test.

4.4 Results

4.4.1 DUSP-1 KO animals show good general health up to 21 months of age

At 21 months of age, most Dusp1 KO, HET, and WT control mice appeared to be generally healthy. A small number of animals demonstrated repeated skin ulcerations and were withdrawn from the trial, or died from unknown causes prior to 21 months of age (Supp. Table 1). Animal weights were taken at 21 months immediately prior to sacrifice to evaluate possible metabolic changes due to loss of Dusp1. Female animals showed no statistically significant difference in weight, however male WT mice were on average 7.8 g heavier (p=0.038) than their KO littermates (Supp. Fig. 1).
4.4.2 DUSP-1 KO animals are not protected from the histological changes associated with spontaneous OA

At 21 months of age, *Dusp1* KO and WT littermate control mice were sacrificed and knee joints harvested for histological evaluation of joint health. Slides of frontally embedded, toluidine blue stained mouse knees were evaluated for OA related histological changes by at least 2 blinded observers, using the OARSI recommended cartilage degeneration scoring system [27]. In both female (n=8) and male (n=5) trials of *Dusp1* KO and WT control mice, no statistically significant differences in joint degeneration were observed in the individually examined joint quadrants, nor the summed total joint score (Fig. 4.1A-D). Similarly, when we compared the scores of male to female cohorts, no significantly different findings were observed between any pairing of groups. In both male and female cohorts, a single animal showed larger than average cartilage degeneration, particularly in the medial tibial plateau (MTP). When these points were assessed as outliers using the Grubbs’ test and excluded, the results noted did not change conclusions on statistical significance, so they remained included for the reported data (Fig. 4.1CD).

To directly evaluate any differences in the articular cartilage thickness in the knee joints of *Dusp1* KO and control WT mice, the distance from the subchondral bone to the surface of the intact articular cartilage was measured. No statistically significant differences were observed when comparing male KO to WT or female KO to WT animals (Fig. 4.2AB).

4.4.3 DUSP-1 KO mice show similar levels of cartilage breakdown products

Following the catabolic activities of various metalloproteinases (MMPs, aggrecanases, etc.) on ECM proteins, neoepitopes are formed at either side of the cleavage site, and can be detected using specialized antibodies. We utilized antibodies for neoepitopes formed through aggrecan and collagen II cleavage to evaluate any differences in the pattern of cartilage degeneration in our *Dusp1* KO mice. The DIPEN neoepitope is formed when the interglobular domain (IGD) of the aggrecan core protein is cleaved by MMPs, and
Figure 4.1 Dusp1 KO mice show similar cartilage damage as controls at 21 months of age. Dusp1 KO and WT control animals were aged to 21 months and right knees were evaluated for cartilage damage following OARSI recommended guidelines. (A-B) Representative toluidine blue stained frontal knee section images of the lateral (Lat.) and medial (Med.) femur (F) and tibia (T) of (A) female (n=8) and (B) male (n=5) mice, least damaged (left) and most damage (right), wild type controls (WT, top) and Dusp1 knockout (KO, bottom). Lesion (red arrowheads) location patterns and size were similar between WT and KO groups. Scale bars = 100 µm. (C-D) Total OARSI cartilage degeneration scores across the lateral tibial plateau (LTP), lateral femoral condyle (LFC), medial tibial plateau (MTP), medial femoral condyle (MFC), and cumulative joint score (Total Joint) of (C) female (n=8) and (D) male (n=5) WT and KO mice. OARSI scores did not show statistically significant differences between WT and KO groups when analyzed by two-way ANOVA with Bonferroni’s multiple comparisons test. Error bars are shown as SEM.
Figure 4.1 Dusp1 KO mice show similar cartilage damage as controls at 21 months of age
Figure 4.2. Dusp1 KO mice show similar articular cartilage thickness when compared to controls. Measurements from the subchondral bone to the intact articular cartilage surface were made at 3 points across the lateral tibial plateau (LTP), lateral femoral condyle (LFC), medial tibial plateau (MTP) or medial femoral condyle (MFC) and averaged. (A) Female (n=8) and (B) male (n=5) Dusp1 KO mice do not show statistically significantly different articular cartilage thickness when compared to WT controls. Analyzed by two-way ANOVA with Bonferroni’s multiple comparisons test. Error bars are shown as SEM. (C) Example diagram demonstrating approximate sites of articular cartilage measurements indicated by dashed red lines. F = femur, T = tibia, Lat. = lateral, Med. = medial.
Figure 4.2 Dusp1 KO mice show similar articular cartilage thickness when compared to controls
represents the new N-terminal of the cleaved aggrecan protein [29]. Likewise, the TEGE neoepitope represents the new N-terminal in the IGD of aggrecanase cleaved aggrecan [30]. Collagen breakdown product neoepitopes can also be similarly identified. The products of MMP mediated cleavage of both collagen I and II form ¾ and ¼ length fragments. The C-terminal of the ¾ fragment is known as the neoepitope C1,2C [31].

No large qualitative differences could be identified when examining immunostained knee sections from either female (n=6, Fig. 4.3A) or male (n=5, Fig. 4.3B) Dusp1 KO and WT control mice. Staining for DIPEN was most pronounced in the matrix of the cartilage lesion, with additional chondrocyte pericellular matrix staining observed in many animals (Fig. 4.3). Weak matrix staining for the TEGE neoepitope was observed in the ECM of cartilage lesions, however little chondrocyte pericellular matrix staining was evident in the MTP (Fig. 4.3). Conversely, increased staining was evident in the pericellular region of chondrocytes in the lateral tibial plateau (LTP) and lateral femoral condyle (LFC) of both male and female animals (data not shown). C1,2C immunostaining was found present only in ECM of the cartilage lesion sites (Fig. 4.3).

Interestingly, the growth plates (tibia and femur) of both female and male animals showed intense DIPEN immunostaining throughout the cartilage matrix (Supp. Fig. 4.2A), but no differences between genotypes were observed. Immunostaining for MMP-13 in the same regions did not appear to be particularly strong, and no differences were noted between WT and KO for either female or male groups (Supp. Fig. 4.2B).

4.4.4 Loss of DUSP-1 causes an imbalance in anabolic but not catabolic chondrocyte activity

The DUSP-1 target ERK mediates the effects of many cell surface receptors including EGFR. Immunostaining for the phosphorylated activated form of ERK (phERK) showed similar staining patterns and intensity in the lateral knee compartment of female Dusp1 KO and WT animals (Fig 4.4A) and males (data not shown). However, little to no cellular staining was observed in the medial compartment of the knee for any group (data not shown).
Figure 4.3. Cartilage matrix neoepitope immunostaining shows similar intensity and localization in Dusp1 KO and WT control mice. (A) Female (n=6) and (B) male (n=5) representative medial femur (F) and tibia (T) joint sections were immunostained for: DIPEN (Top, MMP cleaved aggrecan neoepitope), TEGE (Middle, aggrecanase cleaved aggrecan neoepitope), and C1,2C (Bottom, MMP cleaved collagen II/I). Regions of pericellular and extracellular matrix (ECM) staining are indicated by the black arrowheads. Scale bars = 100 µm.
Figure 4.3 Cartilage matrix neoepitope immunostaining shows similar intensity and localization in Dusp1 KO and WT control mice
Matrix metalloproteinase 13 (MMP-13) cleaves both collagen II (C1,2C neoepitope) as well as aggrecan ECM molecules (DIPEN neoepitope), and has been implicated as one of the key mediators of cartilage destruction in OA [35]. Immunostaining for MMP-13 in knee joint articular cartilage was localized largely to the chondrocyte pericellular ECM and surrounding cartilage matrix immediately adjacent to lesion sites in female (Fig. 4.4B) and male (data not shown) mice. Little difference was noted for MMP-13 staining between WT and KO animals, which is compatible with previous observations for DIPEN and C1,2C neoepitope staining as noted above.

SOX9 is the master regulator of the chondrocyte cellular phenotype, and is important in both establishing a chondrocyte lineage during development as well as maintaining that phenotype in adult tissues [4]. In the lateral compartment of the knee joints of female Dusp1 KO mice the intensity of SOX9 immunostaining was decreased in the articular cartilage chondrocytes and meniscus when compared to WT controls (Fig. 4.4C). Similar results were observed in male animals (data not shown), however, little cellular staining was observed in the medial compartment of either sex (data not shown). The number of SOX9 positive cells in female animals also trended towards a decrease in KO vs WT (p=0.095), but was not found to be statistically significant (Fig. 4.4D).

4.4.5 The gait of DUSP-1 KO mice is comparable to WT mice

To evaluate changes in the gait patterns of our aged Dusp1 KO mice vs WT controls, we utilized the Noldus CatWalk apparatus, which measures parameters of rodent walking patterns. We examined 4 of these parameters, including stride length, duty cycle, print area and paw intensity. No statistically significant differences were found comparing the stride length of the hind limbs of WT vs KO female (Fig. 4.5A) and male (Fig. 4.5B) animals. Similarly, duty cycle (% time in stand phase of walk cycle) was not found to be statistically significantly different between WT vs KO female (Fig. 4.5C) and male (Fig. 4.5D) animals. For both print area and paw intensity, no statistically significant differences were observed between female or male WT vs KO animals when normalized to animal weights (data not shown).
Figure 4.4. Decrease in cartilage anabolism marker SOX9 in Dusp1 KO mice. (A) Representative phosphoERK (phERK) immunostained female (n=6) lateral femur (F) and tibia (T) joint images show similar cellular staining (arrowheads) in Dusp1 KO and WT control mice. (B) Representative MMP-13 immunostained female (n=6) medial femur (F) and tibia (T) joint images show similar pericellular and matrix staining (arrowheads) in Dusp1 KO and WT control mice. (C) Representative SOX9 immunostained female (n=6) medial femur (F) and tibia (T) joint images show decreased staining intensity and positive cells (arrowheads) in Dusp1 KO mice when compared to WT controls. All scale bars = 100 µm. (D) The number of SOX9 positive cells in female Dusp1 KO mice was decreased but not statistically significantly different (p=0.095) from WT controls. Analyzed by unpaired t-test. Error bars are shown as SEM.
Figure 4.4 Decrease in cartilage anabolism marker SOX9 in Dusp1 KO mice
Fig. 4.5. Gait patterns are not different in Dusp1 KO mice at 21 months of age. Gait patterns of Dusp1 KO and WT control mice were measured using the Noldus CatWalk apparatus prior to sacrifice at 21 months of age. (A) Female (n=5) and (B) male (n=5) stride length (distance between individual hind paw prints during gait) were not statistically different between WT and KO mice. (C) Female (n=5) and (D) male (n=5) duty cycle (% of time in stand phase of walk cycle) was also not statistically different between WT and KO mice. Data analyzed by unpaired t-tests. Error bars are shown as SEM.
Figure 4.5 Gait patterns are not different in Dusp1 KO mice at 21 months of age
4.5 Discussion

In this study on the effects of loss of Dusp1 on spontaneous OA in mice, we demonstrated little difference between both female and male Dusp1 KO and WT control mice in terms of OA related cartilage damage and gait changes.

Spontaneous OA can be particularly difficult to study as it may take months to years to develop even in rodent models that develop spontaneous OA-like cartilage damage within 2 years of life [36]. This is in contrast to induced OA models using surgical or chemical methods that model more rapidly developing post-traumatic OA in months or less [36]. Our lab has previously examined mice on the C57Bl/6 background at 21 months of age and has found that they develop mild to moderate OA by this time point (unpublished data). Other studies have shown similar results [37]. While our lab has also shown that F1 C57Bl/6 x 129/SV crossed mice develop mild to moderate OA by 2 years of age [38], future studies should consider using sufficiently backcrossed congenic C57Bl/6 or 129 strain mice to reduce possible confounding genetic variability. However, the severity of OA observed on our mixed background was similar to that seen in our previous work in C57Bl/6 mice.

Previous studies indicate the importance of both ERK and p38 in the progression and initiation of arthritis [8,15] as well as the role of DUSP-1 in the negative regulation of these MAPKs [17,24,39]. Despite this we were unable to show any difference in cartilage degeneration when Dusp1 was genetically removed from our mouse model. This may be in part due to the incredibly complex and dynamic regulation of these particular factors. Both ERK and p38 are regulated at multiple levels (expression, post-translational modification, etc.), with various negative and positive feedback loops, and many different signalling pathways converging on these molecules [10,40]. Therefore, while we may have removed DUSP-1 from our KO mice, the net effect may have been largely mitigated by increased negative feedback or various other factors. Furthermore, compensation by other DUSP protein family members (Reviewed in [17]) may have also counteracted for loss of DUSP-1 in our model, which is consistent with the minimal changes in cartilage phERK immunostaining observed in KO animals. Future studies may need to focus further
upstream at the receptor level, or downstream at specific transcription factors to gain more insight into the pathobiology of OA and related diseases.

Interestingly, in this study we demonstrated a large increase in the immunostaining for MMP cleaved aggregan neoepitope DIPEN in the growth plate of our aged animals. To our knowledge, this has not been previously described. Oddly, MMP-13, which is considered one of the primary proteinases in cartilage ECM breakdown [2,35], did not show particularly robust staining in the same regions. This may be due to the time point at which we examined our animals, particularly if MMP-13 is transiently expressed and the cleaved aggregan fragments are unable to diffuse out of the growth plate due to otherwise intact ECM protein network structure or restriction by surrounding bone.

In this study we demonstrated a small decrease in SOX9 staining with a trend towards fewer stained cells in Dusp1 KO animals, although this was not shown to be statistically significantly different. Previous studies have shown a negative regulatory role for ERK in SOX9 driven chondrogenesis [41,42]. Furthermore, studies in our lab have shown that MEK/ERK inhibition can reverse TGFα/EGFR mediated repression of Sox9 expression [8]. However, regulation of SOX9 is complex and poorly understood, and other studies have shown that ERK increases SOX9 at the mRNA and protein levels [26,43,44], and p38 may stabilize Sox9 mRNA [45]. Since our study did not show a large difference in phERK immunostaining, temporal factors, and other DUSP proteins or unknown pathways may be involved in compensating for loss of DUSP-1 in vivo. Further research is required to elaborate on the role of DUSP-1 and MAPK signalling in the regulation of SOX9.

While the weight of the male control animals was on average higher than the KO group, we did not observe any differences in OA development that is known to be influenced by obesity in humans [2]. It is unknown why the female cohort did not demonstrate a similar difference in weights, but this may be due to differences in hormonal control in males and females that is influenced by ERK signalling and may have been modulated by whole body loss of Dusp1 in this study [46].

While evaluating limb pain in rodent models is difficult to assess and widely debated, analysis of gait changes has begun to gain favour as a measure of behaviours related to
OA-like pain and joint dysfunction [33,34,47]. To evaluate gait changes possibly due to OA development in our study, we utilized the Noldus CatWalk gait analysis system. While we were unable to show any differences between Dusp1 KO and control mice in the gait variables examined, it is still possible that these animals experienced increased OA related changes in gait. Due to the limitations of the equipment, and the nature of spontaneous OA presenting as a bilateral or unilateral disease, any small but present changes in gait pattern may have been lost in the noise. Interestingly, mice have been shown to mask signs of pain with male observers (MP performed all CatWalk data acquisition), possibly making it difficult to assess changes in gait and perhaps OA related pain-like behaviours [48]. Furthermore, it is difficult to determine the cause of gait changes, as muscle weakness due to aging, physical dysfunction of the joint due to cartilage or bone degeneration, and pain all may influence animal gait behaviours.

In conclusion, we were unable to demonstrate any definitive evidence that DUSP-1 plays an important role in spontaneous OA development in mice. However, future research is needed to evaluate if DUSP-1 may play a role in the more rapid progression of cartilage degeneration as seen in post-traumatic OA, and if other DUSP proteins can compensate for the loss of DUSP-1.

4.6 Acknowledgements

The authors are grateful to Dr. J. Mort (Shriner’s Hospital, Montreal) for neoepitope antibodies, and Holly Dupuis, Anusha Ratneswaran, and Dawn-Marie Bryce for their assistance in the blinded scoring of joints. M.A.P. was supported by a CIHR doctoral scholarship. M.A.P. and M.B. were supported in part by the Joint Motion Program a Canadian Institute of Health Research (CIHR) Training Program in Musculoskeletal Health Research and Leadership. F.B. is the recipient of a Canada Research Chair. This work was supported by CIHR Operating Grant MOP 86574 to F.B.
Supplementary Table 4.1. Dusp1 KO mice are generally healthy at 21 months. Dusp1 WT (Dusp1^{+/+}), Het (Dusp1^{+-}), KO (Dusp1^{-/-}) animals live and healthy (Live) at the end of the experiment, or removed from the experiment due to recurrent skin ulcerations/died of unknown causes (Dead/Rem.).
<table>
<thead>
<tr>
<th>Female</th>
<th>Live</th>
<th>Dead/Rem.</th>
<th>% Live</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>9</td>
<td>1</td>
<td>90.0</td>
</tr>
<tr>
<td>Het</td>
<td>8</td>
<td>1</td>
<td>88.9</td>
</tr>
<tr>
<td>KO</td>
<td>9</td>
<td>2</td>
<td>81.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Male</th>
<th>Live</th>
<th>Dead/Rem.</th>
<th>% Live</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7</td>
<td>1</td>
<td>87.5</td>
</tr>
<tr>
<td>Het</td>
<td>5</td>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>KO</td>
<td>7</td>
<td>1</td>
<td>87.5</td>
</tr>
</tbody>
</table>

Supplementary Table 4.1. *Dusp1* KO mice are generally healthy at 21 months.
4.8 Supplementary Figures

**Supplementary Figure 4.1 Male Dusp1 KO are lighter than WT controls.** Animal weights were taken at 21 months prior to sacrifice. Male (n=5) Dusp1 KO mice were on average 7.8 grams lighter than WT controls. Female (n=6) Dusp1 KO and WT mice showed no statistically significant differences in weight. Data analyzed by two-way ANOVA with Bonferroni’s multiple comparisons test. Error bars are shown as SEM.
Supplementary Figure 4.1 Male *Dusp1* KO are lighter than WT controls.
Supplementary Figure 2 Intense DIPEN but not MMP-13 immunostaining in the growth plate of Dusp1 KO and WT mice. Female frontal knee sections were immunostained for (A) DIPEN (MMP cleaved aggrecan neoepitope) and (B) MMP-13. DIPEN staining in both WT and KO growth plates is intense despite poor staining for MMP-13. Scale bar = 100 um. Representative images shown. N=6.
**Supplementary Figure 2** Intense DIPEN but not MMP-13 immunostaining in the growth plate of *Dusp1* KO and WT mice.
4.9 References


Chapter 5

5 Discussion

5.1 Overview

The overall goal of my thesis was to evaluate the role of epidermal growth factor receptor (EGFR) signalling in cartilage biology. I was particularly interested in evaluating the involvement of factors regulating both the EGFR receptor and downstream signalling pathways involved in osteoarthritis (OA), namely mitogen-inducible gene 6 (Mig-6) and dual specificity phosphatase 1 (DUSP-1). My lab has been closely studying the role of EGFR signalling in cartilage biology and pathology, particularly its ligand, transforming growth factor alpha (TGFα), since microarray studies identified it to be increased in cartilage isolated from a surgical rat model of OA [1,2]. Additional studies further demonstrated that TGFα appeared to play a role in the induction of cartilage degeneration and alteration of the healthy chondrocyte phenotype [3,4]. These studies and additional literature demonstrating evidence for EGFR signalling involvement in the alteration of cartilage homeostasis [1–12], encouraged us to explore the various downstream pathways, and regulatory mechanisms that may be in play. Since Mig-6 had been identified as an important regulator of EGFR signalling [13,14], and genetic knockout (KO) studies had shown cartilage degeneration similar to that seen in animal models of OA [9,10], it appeared to be a good candidate for further study into the role of EGFR signalling in cartilage homeostasis.

The first study conducted for my thesis involved the cartilage-specific deletion of Mig-6 (please note that the gene encoding for Mig-6 is Errfi1, however I will use Mig-6 notation for simplicity) in mice, utilizing a Col2a1-Cre mouse [15] to selectively delete Mig-6 from chondrocytes with a recently generated ‘floxed’ Mig-6\textsuperscript{fl/fl} conditional KO mouse obtained from our collaborators [10]. Based on previous findings that TGFα induced EGFR signalling was associated with cartilage degeneration [1–4], and systemic loss of Mig-6 in mice resulted in joint pathology similar to OA [9,10] I hypothesized that cartilage-specific deletion of Mig-6 would lead to premature development of OA in these animals due to overactive EGFR signalling. Interestingly, we found an unexpected mixture of both
anabolic and catabolic phenotypes present in these Mig-6 knockout (KO) animals. The articular cartilage in the knee joints of Mig-6 KO mice demonstrated drastically increased thickness. However, these animals also developed large invasive chondro-osseous nodules in their knee joints and spine similar to previous whole body Mig-6 KO models [9,10]. Interestingly, little evidence of cartilage degeneration was apparent. The central ligaments in the knee joints of KO mice also demonstrated cartilage neogenesis, and were associated with an osteolytic phenotype at the ligament entheses. Tartrate resistant acid phosphatase (TRAP) staining, indicative of osteoclast activity, was increased in the knee entheses, and both subchondral and trabecular bone. It appeared that these phenotypes were associated with increased EGFR activation, and induction of SOX9, for which immunostaining was increased in both the articular cartilage and the chondro-osseous nodules that developed in KO mice. Taken together, it appeared that loss of Mig-6 appears to induce an anabolic phenotype in articular cartilage and produce ectopic chondro-osseous growths, however it also resulted in activation of catabolic activity through osteoclast activation. Thus, I concluded that Mig-6 regulates both anabolic activity in articular cartilage and catabolic activity in surrounding bone.

However, Mig-6 had not been studied in mice past their first year of life when OA generally begins to develop [16], and post-natal modulation of Mig-6 in mice had not been previously attempted, which led into my second study. I again utilized our cartilage specific Mig-6 KO mouse model, and housed these animals until 21 months of age. Examination of the knee, elbow, and ankle joints of Mig-6 KO mice showed increased articular cartilage thickness was present in all of these joints, and could be maintained until 21 months of age near the end of their life span. Furthermore, while KO mice did show increased cartilage matrix loss relative to controls, overall OA-like pathology was surprisingly low. In an additional experiment, I introduced the Tgfa null mutation previously studied in our lab [6,17] to the constitutive Mig-6 KO mice. These mice showed similar formation of chondro-osseous nodules in the knee despite the absence of TGFα. This lead me to conclude that TGFα is not essential for initiating the formation of nodules in Mig-6 KO mice, and that compensation by other EGF-family ligands found in the joint [18,19] may be involved, although other possibilities could explain this phenotype (ex. EGFR-independent effects). I then pursued the post-natal deletion of Mig-6 in mouse cartilage
using a tamoxifen inducible Col2a1-CreER mouse line [20] with the Mig-6fl/fl mice described above. Initiation of CreER mediated Mig-6fl/fl recombination in chondrocytes with tamoxifen at 3 weeks was followed up at 12 weeks of age. Despite previous evidence that constitutive cartilage specific Mig-6 deletion leads to rapid development of cartilage nodules [10,9] and enhanced cartilage thickness by 12 weeks of age, I did not observe similar phenotypes in these CreER-based experiments. I concluded that loss of Mig-6 in cartilage may not be directly responsible for OA-like degeneration previously observed, and may in fact promote long term cartilage health. Furthermore, based on the CreER experiments, I suspect that the phenotype of constitutive Mig-6 KO mice may be at least partially influenced by developmental factors.

Finally, I turned my attention to DUSP-1, a phosphatase that deactivates mitogen-activated protein kinase (MAPK) signalling downstream of EGFR activation [21], and had appeared in our own genome wide studies examining endochondral ossification [22]. Studies in inflammatory forms of arthritis demonstrated that DUSP-1 had a protective effect in animal models [23–25], and thus was a good candidate to assess for its potential role in OA and maintenance of cartilage health. Utilizing a whole body Dusp1 KO mouse line obtained from our colleague [26,27], I initiated a study on spontaneous age-related OA in these animals with the hypothesis that loss of DUSP-1 would lead to dysregulation of signalling downstream of EGFR activation, and would result in exacerbated spontaneous OA. Examining the knee joints of these mice at 21 months of age, I found similar levels of cartilage degeneration in both Dusp1 KO mice and controls. The chondrocytes of KO mice did not appear to show increased levels of phospho-ERK, indicating that loss of DUSP-1 was likely being compensated for by other mechanisms [21]. However, a slight decrease in SOX9 was evident by immunostaining, indicating that DUSP-1 may play a role in regulation of chondrogenic behaviour. With these findings, I concluded that loss of DUSP-1 alone is insufficient to influence age-related spontaneous OA.

Taken together these studies demonstrate that regulation of EGFR signalling plays a complex but important role in the maintenance of joint homeostasis (Fig. 5.1). It is clear, however, that Mig-6 is important for the fine tuning of anabolic activity within articular cartilage, and the entire joint. Furthermore, SOX9 modulation was present in all three
Figure 5.1 Regulation of EGFR signalling in joint homeostasis. Epidermal growth factor receptor (EGFR) signalling influences the homeostasis of multiple tissue types in the synovial joint. Loss of Mig-6 disrupts regulation of EGFR resulting in increased downstream signalling. Resultant increases in SOX9 and ERK activity may induce the formation of abnormal ectopic tissue, and increased anabolic activity within the articular cartilage. Increased EGFR signalling may also enhance osteoclast activity, leading to bone remodeling within the joint. DUSP-1 regulates ERK and p38 signalling that may lead to joint pathology, however this thesis demonstrated that loss of DUSP-1 alone is insufficient to lead to increased cartilage degeneration. ROCK (rho-kinase) activation may also lead to increased cartilage degeneration [4], but additional research is needed.
Figure 5.1 Regulation of EGFR signalling in joint homeostasis
studies, and supports evidence that EGFR signalling may play an important role in the complex regulation of this master chondrocyte transcription factor.

5.2 Contributions and Significance of Findings

5.2.1 Contributions to the Field of Skeletal Biology

Chapters 2 and 3 of this thesis focus on the characterization of Mig-6 KO mice, and the fascinating anabolic and catabolic phenotypes that these animals exhibit. Studies prior to my work showed that systemic loss of Mig-6 resulted in considerable pathology in the form of chondro-osseous growths in a variety of joints including the knee, ankle, and temporomandibular joint (TMJ) [9,10]. Additionally, a study utilizing Prx1-Cre to drive the deletion of Mig-6 in the entire limb with the same Mig-6<sup>fl/fl</sup> line [10] used in my experiments showed similar ectopic growths in the knee joint [28] (this study was published while my work was underway). However, the tissue of origin of these growths was uncertain, as Mig-6 was not present in any of the joint tissues of KO animals. Using chondrocyte-specific deletion of Mig-6, I was one of the first to demonstrate that the cartilaginous tissue of the joints of these KO animals was contributing to these ectopic formations. A similar study was conducted by one of our collaborators and published shortly prior to the publication of our manuscript [29], however, this work was conducted using a novel Mig-6<sup>fl/fl</sup> mouse line [29], and deletion in chondrocytes was driven by a different Col2a1-Cre driver line [30]. Their study showed similar phenotypes in the knee joint, with extensive nodule formation that progressed at a rate similar to my Mig-6 KO mice [31]. Both models did not show any formation of nodules within the TMJ that allowed us to characterize the pathology in the knee joint far longer than previous studies, as our KO mice survived past 3-6 months [9,10]. However, while their study examined the knee joints of cartilage specific KO mice at 15 months of age [29], they did not pursue other joints. Conversely, I have examined the entire calcified skeletons of 36 week old mice via micro-computed tomography (microCT) and shown that the spines of 36 week old Mig-6 KO mice form calcified nodules, but these mice do not appear to exhibit abnormal formation of calcified tissues in other joints. Regardless, these two studies complement
each other well and demonstrate that the findings are not due to artifacts of the genetic models utilized.

In chapter 3, I extend these studies to additional time points and outcome parameters. I describe the formation of nodules in the spine, ribs, and knees of 21 month old cartilage specific Mig-6 KO mice. Similar to data shown in Chapter 2, I closely examined the calcified skeleton of Mig-6 KO mice and did not note the formation of calcified nodules in joints other than the knee and spine. Furthermore, histology of the ankle and elbow confirmed the absence of non-calcified growths in these joints. Additional work is needed to explain the apparent joint specificity observed, however, I have shown evidence that these growths are commonly associated with the supportive ligaments and menisci found in the knee, which establishes a basis for future mechanistic studies.

While the exact mechanism driving the formation of these chondro-osseous nodules in Mig-6 KO animals is unknown, a common theme appears to be the increased presence of activated EGFR within the chondrocyte-like cells that make up these growths [28,29,31] and the articular cartilage [28,29,31,32]. Ligand binding is important for the initial activation of EGFR in our Mig-6 KO animals, and I suggest that an initial mechanical or biochemical insult may be required to initiate pathology. I have observed that some Mig-6 KO animals do not always show bilateral development of nodules in the knee joint, while still exhibiting increased articular cartilage thickness. Impact models of cartilage damage have shown alterations in Mig-6 expression following mechanical injury [33–35], demonstrating that Mig-6 and EGFR are likely sensitive to these types of insult, perhaps due to EGF-family ligand activation as shown with ADAM17 activated amphiregulin and TGFα shedding [36–38]. Along these lines, I am the first to demonstrate evidence that TGFα is not required for nodule development in Mig-6 KO Tgfa null mice. However, further inquiry is required.

Additionally, I demonstrated that strong immunostaining for SOX9 is present in the chondro-osseous nodules in the knee joints of Mig-6 KO mice, and may be driving growth of these neoplasms through expression of a proliferating chondrocyte phenotype. SOX9 has been shown to drive the growth of a number of different sarcomas [39], and so the
model I have established here may prove useful for the future study of benign and malignant tumours of mesenchymal origin.

Interestingly, I showed that post-natal cartilage-specific deletion of *Mig-6* in 3 week old mice, is insufficient to generate the cartilage growths observed in other constitutive *Mig-6* KO models, at least when examined at 12 weeks of age. While additional work is required to examine this effect in detail, previous studies have identified that the cells in the expanded superficial and mid zone cartilage layers of *Prx1-Cre* driven *Mig-6* KO mice express putative markers of resident chondroprogenitors [28,40]. It is possible that these cells are responsible for driving the anabolic growth of articular cartilage and formation of chondro-osseous nodules observed in the various *Mig-6* KO studies [9,10,28,29,32]. Since these cells may not be actively transcribing collagen II at the time point chosen, the *Col2a1-CreER* system used in my post-natal experiments may not have adequately targeted these cells, which may help to explain the lack of obvious phenotype observed in these experiments. Furthermore, evidence of Cre recombination in the synovium has been shown in *Col2a1-Cre* mice despite lack of Cre expression in post-natal tissue, likely due to shared developmental precursors that express *Col2a1* [41]. The nodules that develop in the synovial tissue in classical *Col2a1-Cre Mig-6* KO mice may derive from these cells, and would not be targeted in my *Col2a1-CreER* based experiments, further explaining the lack of phenotype observed.

Finally, the work I present in this thesis is the first to show increased osteoclast activity in the subchondral bone, nodule bone junction, and the entheses of the knee ligaments as a result of cartilage-specific deletion of *Mig-6*. A study utilizing whole body *Mig-6* KO in mice, previously described [9], with additional surgical transection of supportive ligaments to induce instability in the knee joint, demonstrated similar increases in TRAP staining in the subchondral bone 2-4 weeks following surgery in KO animals [32]. However, it was not clear whether this was due largely to loss of Mig-6, or exacerbated by surgical intervention. Furthermore, this study did not examine in detail osteoclast-driven osteolysis of the ligament entheses of the joint. TGFα and EGF induced production of RANKL and subsequent osteoclast recruitment has been shown previously by our lab, and our
colleagues [6,12]. Thus my studies support the notion that EGFR driven signalling is important for induction of osteoclast activity in the knee joint.

5.2.2 Contribution to the Field of Osteoarthritis

In these studies I show that loss of Mig-6 in cartilage causes increased thickness of articular cartilage in the knee, ankle, and elbow that is largely resistant to thinning or degeneration, even in 21 month-old mice. Similar results have been described in the knee up to 15 months in a study using a unique Mig-6$^{fl/fl}$ and Col2a1-Cre driver line discussed above [29]. Previous reports using whole body Mig-6 KO mice have shown rapid thinning and degeneration of cartilage by 4-6 months of age [9,10,28,32], however I would suggest that these effects may be in large part secondary to joint immobilization, instability, and articular cartilage impingement caused by the chondro-osseous nodules that develop in these joints, similar to conditions used in animal models of secondary OA [16]. This view is supported by my data that shows the joints of Mig-6 KO mice that are unaffected by nodule formation, the elbow and ankle, exhibit robust articular cartilage thickness with little evidence of loss of glycosaminoglycan staining. Although there is little published on the use of EGF-family ligands in joint tissue repair, a study showing enhanced in vitro expansion human chondrocytes by EGF [42] may indicate a role for Mig-6 and EGFR signalling in the field of cartilaginous tissue regeneration.

Extensive work in my lab has provided evidence for a role of TGFα in OA, and has shown that TGFα induces the expression of catabolic MMPs and the repression of ECM protein production [1–6,11,43]. Furthermore, HB-EGF stimulation of cartilage shows similar effects [18]. However, the results of my studies have shown quite the opposite phenotype in the articular cartilage, even though activation of EGFR signalling has been shown in the Mig-6 KO model. Similarly, a recent study published by our colleagues demonstrated that pharmacological inhibition and genetic ablation of EGFR in rodent models resulted in increased articular cartilage destruction, supporting the role of EGFR in the maintenance of cartilage health during OA [44]. These apparent opposing roles are difficult to reconcile, however, regulation of EGFR is highly complex, and downstream signalling pathways may be highly ligand and context specific [45–47]. Mig-6 is important in EGFR desensitization and receptor internalization, a highly complex process that is important for the complete
activation of certain EGFR signalling pathways, and may also be important in the function of EGFR as a putative nuclear receptor [48–50]. Therefore while I have shown increased EGFR activation with loss of Mig-6, the disruption of normal intracellular trafficking may have interesting consequences that are not evident when EGFR is activated by increased environmental ligand alone.

This is the first report on the role of DUSP-1 in long term cartilage health and spontaneous OA. While we did not show significant differences between KO and control animals in terms of cartilage degeneration, these data do provide evidence that there may be other mechanisms present in articular cartilage that compensate for the loss of MAPK regulation by DUSP-1. In fact, DUSP-1 deactivates both p38 and ERK proteins [21], which may have opposing effects in cartilage health [4,8,51–55]. Blockade of ERK signalling has been shown to ameliorate cartilage degeneration both in vitro and in vivo [4,8], while p38 inhibition in vivo exacerbates cartilage degeneration via induction of catabolic enzymes like MMP-13 [51]. However, there seems to be conflicting evidence available for both of these MAPK pathways [4,52–55]. Likewise, MAPK pathway mediated regulation of SOX9, which is important in maintenance of the articular chondrocyte phenotype, is also highly complex and is currently fraught with apparent contradictions [4,31,56–58]. Considering this, it is possible that in my study the effect of loss of DUSP-1 on both p38 and ERK is partially opposing and results in only a slight net decrease in SOX9 and a negligible change in MMP-13 immunostaining in KO tissues. Furthermore, while I also showed an increase in SOX9 immunostaining in the articular cartilage of Mig-6 KO animals, the precise mechanism is unknown, for similar reasons as presented above. Therefore, additional research is required to elucidate the role of EGFR and MAPK signalling in the regulation of SOX9 and cartilage degeneration in OA.

5.3 Limitations of Research

5.3.1 Limitations of in vivo models

As with any study there are limiting factors that may have influenced the conclusions that I have come to. The single largest limiting factor in this thesis is perhaps also one of its greatest strengths, the holistic in vivo nature of my mouse models. These models have
allowed me to evaluate the role of Mig-6 and DUSP-1 in the context of the whole joint, with all the systemic, biomechanical, and environmental factors intact. However, we are inherently unable to completely control for all variables involved including: animal behaviour (fighting, activity, etc.), genetic drift [59], housing conditions, litter size, and likely many additional unknown factors. These effects may have been amplified by the long term nature of some of my experiments. However, I attempted to account for some of these issues by utilizing littermate control animals housed together with their respective KO siblings wherever possible.

The relatively low N value for some experiments conducted may have impacted the validity of some conclusions. The 21 month aging studies for constitutive cartilage-specific Mig-6 KO may have been particularly susceptible with an N of only 3 for many evaluations made. To increase the N for some experiments particularly vulnerable to variability and noise within the data, I utilized an additional pair of age matched controls, and noted this in the appropriate locations of the chapter. However, despite the low N value, I was able to show significant differences between KO and control animals in a number of variables assessed, which further emphasizes the magnitude of these differences. Additionally, Mig-6 KO Tgfa null experiments utilized an N of 2 female, and 1 male trials. While I have observed similar phenotypic development in both male and female animals, it is possible that the mixed sex of the animals used in this experiment influenced the findings. Experiments are under way to increase the N for all of these studies, but due to the long duration of the studies and the complex breeding for double KO mice, results won’t be available for several months. Catwalk generated gait data proved particularly difficult to obtain ‘clean’ results. Previous studies using this system utilized a number of animals nearly double the size of many of my experiments, and still suffered from large variability within the data [60]. Therefore, future studies may need to drastically increase the number of animals used, if reliable gait data is to be evaluated using this system. However, in general we have not seen strong effects in this version of the CatWalk system (Noldus CatWalk 7.1) using multiple models of OA (Beier lab, unpublished).

I heavily utilized chemical staining and immunostaining techniques throughout the studies presented in this thesis. While these techniques are extremely useful for the visualization
of tissue damage, morphology and so on, they are difficult to quantify accurately. I made every attempt to ensure that the histological techniques used accurately present the pathology described, however additional molecular techniques (qPCR, Western Blotting, etc.) may have revealed additional tissue effects missed by my methodology. In upcoming follow-up work, I intend to employ these molecular techniques whenever possible.

The tamoxifen inducible Col2a1-CreER [20] system I utilized to selectively delete Mig-6 [10] from chondrocytes is inherently limited by its ability to express the CreER fusion protein only while Col2a1 is being actively transcribed by the chondrocytes. This means that recombination efficiency may have been affected at the age we chose to deliver tamoxifen (21 days of age, weaning), despite evidence indicating adequate recombination in articular chondrocytes in mice 1 month of age using a similar injection protocol [20]. This age was chosen for a variety of practical reasons, as mothers tend to reject young mice injected prior to weaning (personal observation), and animals at this age tend to tolerate intraperitoneal tamoxifen injections well. I am currently in the process of repeating these experiments using a tamoxifen inducible Agc1-CreER(T2) mouse line [61] that drives efficient CreER expression and subsequent recombination in articular chondrocytes transcribing the aggrecan gene, even in 6 month old animals.

The constitutive cartilage-specific Mig-6 KO mice were established by using the Col2a1-Cre [15] and Mig-6$^{0/0}$ [10] described in detail above. The nature of this type of tissue-specific KO necessitates that recombination occurs during embryonic growth, resulting in deletion of Mig-6 during development as well as in adult tissue. While it is possible that this influenced the development of the bones and joints of KO mice, I did not observe significant differences in the long bone lengths of mice at 40 days of age, and previous studies have not described skeletal growth phenotypes [9,10,28,29,32]. The Col2a1-CreER experiments were conducted in part to address these possible effects.

Recent work utilizing classical and inducible Cre inserted directly into the endogenous collagen X gene (Coll10a1) in combination with various reporter systems has demonstrated that during the process of endochondral ossification, a subset of hypertrophic chondrocytes contribute to the osteoblast pool, perhaps through a trans-differentiation
mechanism [62–65]. This may have serious implications for the ‘cartilage specificity’ of our Col2a1-Cre driver line as at least some of the osteoblasts, and descendent osteocytes, contributing to trabecular bone will exhibit recombination and could thus be deficient in Mig-6 [66]. These findings may affect the interpretation of osteoclast activity shown by increased TRAP staining in the trabeculae of Mig-6 KO mice in Chapter 2, which may have been influenced by loss of Mig-6 in the resident osteoblasts and osteocytes. However, based on these studies my data does support findings that show that increased EGFR signalling in osteoblasts increases the recruitment of osteoclasts [6,19]. Similarly, a recent study has also shown that a Col2a1-Cre driver similar to the one used in my studies induces recombination in the knee joint synovium [41]. As I have observed ectopic growths in joint regions associated with the synovium in Mig-6 KO mice, it is possible that these non-cartilaginous tissues contribute to the formation of ectopic nodules in the knee joint. Further research is still needed to establish the exact tissue(s) of origin for ectopic nodules using my mouse models, although my lineage tracing experiments (Chapter 3) suggest that the nodules are derived from cells expressing Cre in a cell-autonomous manner. Similar to studies described above, we are in the process of increasing the N for these experiments to obtain conclusive evidence for this.

Unfortunately, I was unable to directly demonstrate that Mig-6 protein is not present specifically in targeted KO tissues, as efforts to utilize a Mig-6 antibody for immunostaining have largely failed in my hands. PCR evidence of recombination was certainly contaminated by tissues not expressing Col2a1 and is simply a confirmation that at least some recombination is occurring. Supplementary data provided using a ROSA26\textsuperscript{mTmG} dual reporter system provides some evidence that CreER mediated deletion is taking place in the chondrocytes of CreER(+) mice injected with tamoxifen, but is severely limited by an N value of 1.

While my studies and others demonstrate evidence for the role of EGFR signalling mediating the results observed in Mig-6 KO mice [28,29,32], I cannot be sure that this is due exclusively to Mig-6—EGFR interactions. Mig-6 binds to a number of candidate proteins that may have contributed to the phenotypes described including: all 4 of the EGFR family receptors (ErbB1-4), MET, c-Abl and Cdc42 [67,68]. However, little is
known about the overall effects of Mig-6 interactions with these alternative binding partners, particularly their roles in cartilage in vivo [67,68], and so additional work is required to elucidate their function in the phenotypes I have described.

The whole body Dusp1 KO mice utilized in my studies were generated through the insertion of the commonly used neomycin cassette into the Dusp1 gene, disrupting exon 2 and reducing DUSP-1 protein expression to undetectable levels [26]. Since the deletion of Dusp1 affects all tissues in the body of these animals, it is possible that loss of DUSP-1 protein in various tissues may have influenced hormonal signalling, muscle function (important in joint alignment and gait analysis), metabolism, behaviour, or the immune system in unexpected ways that may have affected disease progression in KO mice. In addition, since Dusp1 is also constitutively removed during embryonic stages, the development of Dusp1 KO mice may have been altered in such a way that the clinical relevance may be influenced as discussed above.

Furthermore, a report that Dusp1 KO mice develop spontaneous osteolytic disease in the toes at approximately 20-24 months of age [24] may indicate that pathology unrelated to OA may have been captured in our experiments. Gait analysis in particular would be susceptible to misinterpretation of results. However, we did not observe any obvious evidence of toe swelling in a colony of greater than 20 KO animals over the course of this study. Overall, the fact that aging-associated osteoarthritis did not appear to be altered in the Dusp1 KO mice suggests that global deletion of the gene did not induce systemic effects influencing our findings.

5.4 Future Directions

A number of future studies could be established to expand on, and further elucidate the findings of this thesis. While I have shown a number of Mig-6 KO related phenotypes in vivo, these results need to be scrutinized in an in vitro environment where additional variables can be adequately controlled for. In particular, we still lack sufficient data to conclusively describe a mechanism for both anabolic growth of chondro-osseous nodules, and increased articular cartilage thickness observed in my Mig-6 KO mice. With this in mind, there are a number of different proteins that interact directly or indirectly with Mig-
6 (ex. MET, c-Abl, Cdc42) and may be partially responsible for the phenotypes I have described. Interaction between these factors and Mig-6 could be examined using in vitro cell and tissue culture methods with cells isolated from Mig-6fl/fl to further elucidate a mechanism for chondrocyte proliferation and activation of cartilage neogenesis and ossification. These studies will also help to reveal which of the phenotypes in our mutant animals are due to altered EGFR signalling, and which are due to other pathways. Similarly the viability of Mig-6 and EGFR signalling pathways for use in expanding chondrocytes, or ex vivo cartilage tissue itself for use in cartilage regenerative techniques could also be examined.

Early attempts to utilize Mig-6 KO mice for use in experiments based on surgical induction of OA [16] failed due to the formation of ectopic tissue physically blocking surgical access to the joint. While I did not observe any notable phenotypes in heterozygous Mig6fl/+ Col2a1-Cre+/- mice during my studies (data not shown), these animals may respond in an interesting fashion to models of post-traumatic OA. Based on the findings of this thesis, these animals may show protection from cartilage degeneration in these models, and perhaps with greater incidence of bony osteophyte formation. Regardless, these studies would help to evaluate the potential of Mig-6 and EGFR signalling as therapeutic targets in post-traumatic OA. In addition, conditional KO in the cartilage just prior or after surgery would be interesting. Given the limitations of the Col2-CreER driver described above, we have bred our conditional Mig-6 KO mice with the Age1-CreER(T2) mice [61] that confer more robust recombination in postnatal articular cartilage. This strategy will permit the use of the DMM surgical model as well as allow for more detailed functional studies on the development of the ectopic nodules.

Tissue from human diseases exhibiting similar pathological features (ex. enchondromas, osteochondromas [69]) as the ectopic nodules in my Mig-6 KO mice, would be valuable to examine for Mig-6 mediated effects. However, the rarity of these diseases and difficulty obtaining samples may make this line of study difficult.

Furthermore, while I have performed a number of Mig-6 loss of function studies described in this thesis, gain of function studies could further elucidate the function of Mig-6 and
EGFR signalling in cartilage. In fact, a conditional *Mig-6* overexpressing line of mice has recently been developed that would be well suited for both *in vivo* and *in vitro* study designs [70].

While I have evaluated the role of DUSP-1 in spontaneous OA and found its influence to be largely lacking, the role of this protein in post-traumatic OA has not been assessed. These studies may prove particularly interesting as DUSP-1 appears to be closely involved with inflammation [23–25] that appears to play a key role in the formation of post-traumatic OA [71]. Furthermore, additional DUSP proteins could be examined for their role in cartilage homeostasis *in vitro* and *in vivo* [21]. DUSP-5 in particular appears to act similarly to DUSP-1 in an inflammatory model of arthritis, and may be an interesting target for future studies [72].
5.5 References


11. Hallbeck AL, Walz TM, Briheim K, Wasteson Å, Wasteson A, Wasteson Å. TGF-
alpha and ErbB2 production in synovial joint tissue: increased expression in arthritic

receptor (EGFR) signaling regulates epiphyseal cartilage development through β-
doi:10.1074/jbc.M113.463554

13. Zhang X, Pickin K a, Bose R, Jura N, Cole PA, Kuriyan J. Inhibition of the EGF
receptor by binding of MIG6 to an activating kinase domain interface. Nature.
2007;450: 741–4. doi:10.1038/nature05998

regulation of the EGFR-MAPK cascade by actin-MAL-mediated Mig6/Errfi-1

Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the
doi:10.1083/jcb.200302066

16. Fang H, Beier F. Mouse models of osteoarthritis: modelling risk factors and
21. doi:10.1038/nrrheum.2014.46

17. Mann GB, Fowler KJ, Gabriel A, Nice EC, Williams RL, Dunn AR. Mice with a
null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair,
doi:10.1016/0092-8674(93)90227-H

factor-like growth factor (HB-EGF) is increased in osteoarthritis and regulates
chondrocyte catabolic and anabolic activities. Osteoarthr Cartil. Elsevier Ltd; 2015;
doi:10.1016/j.joca.2015.04.019

19. Schneider MR, Sibilia M, Erben RG. The EGFR network in bone biology and

20. Zhu M, Chen M, Lichtler AC, O’Keefe RJ, Chen D. Tamoxifen-inducible Cre-
recombination in articular chondrocytes of adult Col2a1-CreER(T2) transgenic

reviews3009.1. doi:10.1186/gb-2002-3-7-reviews3009


60. Parvathy SS, Masocha W. Gait analysis of C57BL/6 mice with complete Freund’s adjuvant-induced arthritis using the CatWalk system. BMC Musculoskeletal Disorders. BMC Musculoskeletal Disorders; 2013;14: 14. doi:10.1186/1471-2474-14-14


Appendices

Appendix A: Animal Use Protocol

**AUP Number:** 2015-031

**PI Name:** Beier, Frank

**AUP Title:** Regulation Of Endochondral Bone Growth By Hormones

**Approval Date:** 07/10/2015

**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.2015-031::1

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
Appendix B: Permission to Include Published Manuscript

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS
Aug 06, 2015
This Agreement between Michael A Pest ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number 3683350890379
License date Aug 06, 2015
Licensed Content Publisher John Wiley and Sons
Licensed Content Publication Arthritis & Rheumatology
Licensed Content Title Disturbed Cartilage and Joint Homeostasis Resulting From a Loss of MitogenInducible Gene 6 in a Mouse Model of Joint Dysfunction
Licensed Content Author Michael A. Pest, Bailey A. Russell, YuWen Zhang, JaeWook Jeong, Frank Beier
Licensed Content Date Sep 26, 2014
Pages 12
Type of use Dissertation/Thesis
Requestor type Author of this Wiley article
Format Print and electronic
Portion Full article
Will you be translating? No
Title of your thesis / dissertation
EGFR Signalling in Osteoarthritis and Joint Homeostasis
Expected completion date Sep 2015
Expected size (number of pages) 200
Billing Type Invoice
Attn: Michael A Pest

Total 0.00 CAD
Curriculum Vitae

Michael Andrew Pest

Education

PhD Pharmacology and Toxicology
2010 - Present
The University of Western, Ontario, London, Ontario

BMSc (Honors) Specialization in Pharmacology
2005 - 2010
The University of Western Ontario, London, Ontario

Publications

Pest MA, Pest CA, Bellini MR, Beier F. Deletion of Dual Specificity Phosphatase 1 Does Not Predispose Mice to Increased Spontaneous Osteoarthritis. PLOS ONE, Submitted

Usmani SE, Pest MA, Ulici V, Hill T, Welch I, Mort JS, Beier F. Context-specific protection of TGFalpha null mice from osteoarthritis. Disease Models and Mechanisms, In Revision


Awards and Scholarships

- Schulich Graduate Scholarship, Sept. 2010 – Sept. 2015
  Schulich School of Medicine and Dentistry, Western University – Canada
- CIHR Banting/Best Doctoral Award, April 2012 – April 2015
  Canadian Institutes of Health Research
- CIHR JuMP Program Award, Held Sept. 2010 – April 2015
  Joint Motion Program, Western University – Canada
- Dr. Suzanne Bernier Memorial Award in Skeletal Biology, May 2015
  Schulich School of Medicine and Dentistry, Western University – Canada
- Lucille & Norton Wolfe LHRD Trainee Publication Award, April 2015
  Schulich School of Medicine and Dentistry, Western University – Canada
- Hari and Gudrun Sharma Award, Oct. 2014
  Schulich School of Medicine and Dentistry, Western University - Canada
- CAN Graduate PhD/Pfizer Award, Held July 2011 – May 2012
  Canadian Arthritis Network/Pfizer, Toronto, Ontario
- Graduate Student Travel Award, Held April 2012 – Sept 2012
  Division of Experimental Oncology, Western University – Canada
- Schulich Graduate Thesis Research Award, Held May 2011 –Sept 2012
  Schulich School of Medicine and Dentistry, Western University – Canada
- CIHR Joint Motion Program (JuMP) Scholarship, Held Sept 2010 – July 2011
  Joint Motion Program, Western University - Canada

Career Development

CIHR Joint Motion Student Seminar Committee, 2011 – 2013
CIHR Joint Motion Program, University of Western Ontario

Ivey School of Business Health Sector Leadership Training Program, 2010 – 2013
Ivey School of Business - CIHR Joint Motion Program, University of Western Ontario
Teaching

Teaching Assistant – Physiology 4530: Skeletal Physiology
Jan. 2013 – April 2015
Western University – Canada, London, Ont.


- Training of two Undergraduate Honors Thesis Students, 2012-2014
- Training of one Medical School Student Summer Research Training Program, 2012-2013

Scientific Meetings/Presentations

Oral Presentation

Oral Presentation

Oral Presentation

Poster Presentation

Oral Presentation
Oral Presentation

Oral Presentation

Poster Presentation

Poster Presentation

**Pest, M.,** Pitelka, V., Ohora, S., Ratneswaran, A., Beier, F. The effects of delayed administration of Rho-kinase inhibitor Fasudil on surgically induced Osteoarthritis is Rats. Canadian Connective Tissue Conference. Toronto, Ont. June 2012
Poster Presentation

**Pest, M.,** Pitelka, V., Ohora, S., Ratneswaran, A., Beier, F. The effects of delayed administration of Rho-kinase inhibitor Fasudil on surgically induced Osteoarthritis is Rats. OARSI 2012. Barcelona, Spain. April 2012
Poster Presentation

Poster Presentation

**Pest, M.,** Pitelka, V., Beier, F. The effects of delayed administration of Rho-kinase inhibitor Fasudil on surgically induced Osteoarthritis in Rat. Canadian Arthritis Network Annual Scientific Conference. Quebec City, Quebec. Oct 2011
Poster Presentation