This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.
Investigating the role of \textit{Wt1} in bone and marrow biology

Sophie L. McHaffie

PhD by Research
University of Edinburgh
2014
Declaration

This thesis has been entirely composed by me. Apart from where stated the experiments were carried out solely by me. The work has not been submitted for any other degree or professional qualification.

Sophie L. McHaffie, February 2014
Abstract

The bones of the body vary in size and shape, but are fundamentally all composed of the same cell types: osteoblasts, osteoclasts, osteocytes, vascular cells, and sometimes marrow cells. Long bones are formed when mesenchymal stem cells (MSCs) give rise to chondrocytes i.e. cartilage cells, and osteoblasts i.e. bone cells. These develop to form layers of bone encasing a cartilagenous core which eventually becomes the marrow cavity. A recent study showed that deleting the *Wilms’ tumour gene, Wt1*, in adult mice causes a dramatic loss of bone and fat tissue, fat being another derivative of MSCs. This finding led me to ask whether *Wt1* expression is involved in bone biology and whether it plays a functional role in the stem or progenitor populations.

*Wt1* is a transcription factor that acts as a mesodermal / mesenchymal regulator. It acts as a tumour suppressor gene with mutations leading to the eponymous paediatric kidney tumour. However, in adult cancers it has oncogene characteristics, being highly expressed in the tumours of tissues in which it is not normally present. It also plays a pivotal role in the epithelial to mesenchymal transition (EMT) and vice versa in developing heart and kidney, respectively. There is, however, no evidence of its involvement with EMT / MET in adults. *Wt1* is expressed in various developing tissues and is particularly vital for kidney development. Due to its involvement as a regulator of EMT / MET during development and the phenotype observed following its deletion *in vivo*, we hypothesised that *Wt1* is expressed in, and required for the function of mesenchymal stem or progenitor cells populations within the bone marrow.

A *Wt1*-GFP knock in mouse was used to show that *Wt1* expressing cells are found in the bone marrow, and also for the first time in the bone. The GFP population overlaps with a non-haematopoietic MSC population defined by 3 cell surface markers in the bone and marrow, as well as an osteoblast (OB) progenitor population. Using a tamoxifen inducible CreERT2 showed that *Wt1* loss alters the proportion of GFP cells in the bone and marrow cells that overlap with these MSC
and OB progenitor markers, but microarrays were needed to assess the functional effects of *Wt1* deletion.

Microarrays highlighted various pathways that were altered following the *in vitro* deletion of *Wt1* in total bone and marrow culture, as well as the non-haematopoietic GFP⁺ and GFP⁻ populations. In bone cells, deleting *Wt1* negatively affects various pathways related to MSCs and their derivatives, including collagen biosynthesis, cartilage development and muscle tissue development. Also negatively affected were Wnt signalling regulation and EMT regulation; this is the first time *Wt1* has been shown to be involved in EMT in adult cells. These findings were validated using qRT-PCR to show the down regulation of various genes involved in each pathway, showing that as well as being expressed in these populations it is also playing a functional role. Ossification pathways were negatively altered in the cells not expressing *Wt1* following the deletion of the gene suggesting that *Wt1* may also be acting in a paracrine manner to play its role in bone homeostasis.

As well as in adult tissues, *Wt1* was found to be expressed during development in the limb tissue of e11.5 to e16.5 mice. Preliminary results show that *Wt1* may also have a functional role during bone development, as loss of expression causes a reduction in the percentage of non-haematopoietic MSC cells in the e18.5 hindlimb. As well as this, preliminary lineage tracing experiments suggest that cells found at the bone surface are of *Wt1*⁺ origin.

This thesis has also highlighted the importance of experimental conditions and controls, particularly for CFU-F assays. CreER², *loxP* sites, tamoxifen, oxygen tension levels, and gender all exert specific effects on colony formation, independent of *Wt1* expression.

In conclusion, these data identify *Wt1* as a key player in bone development and homeostasis. The microarray results led to the conclusion that *Wt1* has a functional role in several mesenchymal pathways and highlights various genes that are potential *Wt1* targets and should be further investigated using ChIP-Seq methods.
Acknowledgments

Firstly I would like to thank my supervisors who have given me so much encouragement and help over the last few years. Thank you to Nick for your continual support, enthusiasm, and humorous charade playing. Thank you to You-Ying for not just being the best supervisor throughout my PhD, but also a really lovely friend, supplier of chocolate, and cocktail advisor. I’m raising a Caipirinha to you sweet pea!

Thank you to the lovely Hastie Lab, past and present, for making it a happy place to work, and especially to Rachel for her immuno wizardry. I’m so grateful to everyone who has helped me in different ways throughout my PhD: My thesis committee (John, Julia, and Rob); Anna, Davy, and John for your mouse help; Graeme for being a (very patient) bioinformatics whizz; Craig for beautiful graphics; Paul and Matt for un-jinxing the microscopes for me; all the technical staff for EVERYTHING; Lizzie for FACSing hundreds (thousands?) of tubes of cells; and Katie, Brenda, and Diane for keeping me in order!

Thank you to my C4 office mates, especially Fay, Iain, Victor, honorary member Sarah, and the creepy Cliff Richard calendar (why wasn’t it David Tennant again!?).
Thank you also:

To Axel for all your encouragement and advice over the years and for getting me onto this particular path of science.

To everyone at South Lakes for all your love and a rucksack that lasted me my entire University life, and especially to Mike for listening to my scientific ramblings.

To my un-sciencey friends, especially the Games Nighters (notably tiers 1 & 2 ;-))

To Cam who thinks I’m a midwife, and Alison, my lovely gin and Harry Potter friend.

A huge thank you to my entire family: my wonderful mum Mandy and brother Sam for their continual love and support; Jan and Pete for picking me up and feeding me after long lab days; and my lovely Grandma for always believing in me.

And finally, though he can't possibly know how much of a help he has been, I'd like to thank my husband Ben who has always encouraged me towards excellence and Squiggly for all her late night puppy cuddles.
Table of Contents

Declaration  ii  
Abstract  iii  
Acknowledgments  v  
Table of contents  vii  
List of Figures  xii  
List of Tables  xvi  
List of Abbreviations  xviii

Chapter 1: Introduction

1.1 Bone Formation  2

1.2 Bone Marrow Overview  6
  1.2.1 Bone marrow stroma is a heterogeneous population  6
  1.2.2 Vascularisation is vital for bone marrow formation  7
  1.2.3 Bone marrow stromal cells have conventional and unconventional plasticity  9

1.3 Mesenchymal Stem/Progenitor Cell definition  11
  1.3.1 Stem cells as a therapeutic tool  17
  1.3.2 Mesenchymal stem cell niche within the bone and marrow  19
  1.3.3 Mesenchymal Progenitors  22
    1.3.3.1 Osteoblast progenitors – CD166 (ALCAM) and Sca-1  23
    1.3.3.2 Chondrocyte progenitors – CD29 and CD146  25

1.4 Overview of Wt1 – a mesodermal / mesenchymal regulator  26
  1.4.1 Wt1 in development  30
  1.4.2 Wt1 in adult homeostasis  32
  1.4.3 Wt1 and oxygen levels  33
  1.4.4 Wt1 and IGF1  36
  1.4.5 Wt1 and chemokines  36
  1.4.6 Wt1 in tissue damage and repair  37
  1.4.7 Wt1 and MSCs  39

1.5 Summary  40
  1.5.1 Hypothesis  41
  1.5.2 Aims  41
Chapter 2: Materials & Methods

2.1 General Reagents

2.2 Transgenic Animal Models

2.2.1 Animal Husbandry

2.2.2 Genotyping of mice

2.2.3 WER mouse line

2.2.4 Wt1-GFP mouse line

2.2.5 WGER mouse line

2.2.6 Wt1\textsuperscript{CreERT2}:mTmG mouse line
  \hspace{1cm} 2.2.6.1 In Vivo lineage tracing

2.2.7 Cre control mouse line

2.2.8 In Vivo deletion

2.2.9 In Utero deletion

2.3 Tissue Culture

2.3.1 Isolation and expansion of murine MSCs

2.3.2 Adipogenic differentiation

2.3.3 Osteogenic differentiation

2.3.4 Chondrogenic differentiation

2.3.5 Colony forming unit fibroblast assay

2.4 Immunohistochemistry

2.4.1 Sample preparation

2.4.2 H&E staining

2.4.3 Solutions for paraffin immunoperoxidase and immunofluorescence

2.4.4 Immunoperoxidase – paraffin sections

2.4.5 Dual Immunofluorescence – paraffin sections

2.4.6 Imaging

2.5 Electrophoresis

2.5.1 Gel electrophoresis of DNA samples

2.6 Gene Expression Analysis

2.6.1 RNA Extraction
  \hspace{1cm} 2.6.1.1 RNA Isolation using TRIzol\textregistered Reagent
  \hspace{1cm} 2.6.1.2 RNA Isolation and purification using Qiagen RNeasy Mini Kit
  \hspace{1cm} 2.6.1.3 RNA Isolation using Arcturus\textregistered PicoPure\textregistered RNA Isolation Kit
  \hspace{1cm} 2.6.1.4 RNA quality and quantity

2.6.2 cDNA Synthesis

2.6.3 Quantitative Realtime polymerase chain reaction (qRT-PCR)

2.6.4 Custom qRT-PCR plates
2.7 Flow Cytometry: Fluorescence Activated Cell Sorting (FACS) 60

2.8 Gene Expression Microarrays 61
   2.8.1 Microarray sample preparation – cRNA amplification 61
   2.8.2 Gene Expression 61
   2.8.3 Gene Expression Analysis 62

2.9 Statistical Analysis 62

Chapter 3: Characterisation of Wt1+ population in bone and bone marrow

3.1 Introduction 64

3.2 Characterising the GFP positive population in vivo and in vitro 64

3.3 Relating the Wt1-expressing GFP population to an MSC population 68
   3.3.1 In Vivo 68
   3.3.2 In Vitro 73

3.4 Relating the GFP population to osteoblast progenitor markers 78
   3.4.1 In Vivo 79
   3.4.2 In Vitro 80

3.5 Discussion 86

Chapter 4: Testing the stem/progenitor properties of the Wt1+ cell population in adult bone and bone marrow

4.1 Introduction 91

4.2 In Vitro differentiation capabilities of GFP positive cells 91

4.3 In Vitro adipogenesis and Wt1 92

4.4 Colony Forming Unit Fibroblast Assay (CFU-F) 95

4.5 Cre toxicity effects 101

4.6 Lineage Tracing 105
   4.6.1 In Vivo Lineage Tracing 106

4.7 Discussion 107
Chapter 5: Pathways affected by Wt1 deletion in bone and bone marrow

5.1 Introduction

5.2 Total cell microarrays
   5.2.1 GO terms down regulated in Wt1 deletion mutants
   5.2.2 GO terms up regulated in Wt1 deletion mutants
   5.2.3 GO terms altered by oxygen level

5.3 Cre control microarrays

5.4 Gene expression validation

5.5 Lin-CD31-GFP sorted microarrays
   5.5.1 GO terms down regulated in Wt1 mutant GFP+ cells
   5.5.2 GO terms up regulated in Wt1 mutant GFP+ cells
   5.5.3 GO terms enriched in Wt1 mutant GFP- cells
   5.5.4 GO terms enriched in control GFP+ cells

5.6 Discussion

Chapter 6: Wt1 expression during bone development

6.1 Introduction

6.2 GFP positive cells are present in developing limbs

6.3 The effect of Wt1 deletion on bone development

6.4 Osteoblast progenitors during development

6.5 Lineage tracing during development
   6.5.1 Induction at e14.5 and analysis at e18.5
   6.5.2 Induction at e14.5 and analysis at 10 days
   6.5.3 Induction at e14.5 and analysis at 3 weeks

6.6 Discussion

Chapter 7: Discussion and Future work

7.1 Introduction

7.2 Wt1 expressing cells in the bone and marrow
   7.2.1 The effect of Wt1 deletion on total cell genetic expression patterns

x
7.2.2 The effect of Wt1 deletion on non-haematopoietic GFP+ genetic expression patterns 192
7.2.3 The effect of Wt1 deletion on non-haematopoietic GFP- genetic expression patterns 192
7.2.4 The expression pattern differences between non-haematopoietic GFP+ and GFP- cells 193

7.3 Wt1 expression and hypoxia 193
7.4 Wt1 expressing osteoblast progenitors 195
7.5 The effect of activated CreER recombinase 196
7.6 A sex effect on colony forming abilities 198
7.7 The effect of inactivated CreER recombinase 199
7.8 Tracing the lineage of Wt1 expressing cells 200
7.9 Wt1 expression during development 200
7.10 Future Work 202
7.11 Conclusions 203

References 205

Appendix

Appendix 1: CFU-F assay – Schematic diagram 224
Appendix 2: Flow cytometry plots for gating strategies 225
Appendix 3: Osteoblast progenitor quadrant controls 227
Appendix 4: Total cell microarray – Schematic diagram 228
Appendix 5: Sorted cell microarray – Schematic diagram 228
Appendix 6: Lineage tracing representative flow cytometry plots 229
## List of Figures

### Chapter 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Bone formation in a mouse tibia</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>A schematic outline of the mesengenic process</td>
<td>12</td>
</tr>
<tr>
<td>1.3</td>
<td>The haematopoietic stem cell niche</td>
<td>20</td>
</tr>
<tr>
<td>1.4</td>
<td>The location of the HSC niche in trabecular bone cavities</td>
<td>21</td>
</tr>
<tr>
<td>1.5</td>
<td>The WT1 locus found on chromosome 11 (band p13)</td>
<td>27</td>
</tr>
<tr>
<td>1.6</td>
<td>The cyclical nature of EMT and MET</td>
<td>28</td>
</tr>
</tbody>
</table>

### Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>The RealTime ready Custom Plates</td>
<td>59</td>
</tr>
</tbody>
</table>

### Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td><em>In Vivo</em> Wt1 mRNA Expression</td>
<td>66</td>
</tr>
<tr>
<td>3.2</td>
<td>Percentage of GFP(^+) cells found in MSC cultures increases under</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>hypoxic conditions</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td><em>In Vitro</em> Wt1 mRNA expression</td>
<td>67</td>
</tr>
<tr>
<td>3.4</td>
<td><em>In Vivo</em> Deletion of Wt1 has no effect on the bone marrow</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>sub populations</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td><em>In Vivo</em> Deletion of Wt1 has no effect on the bone sub populations</td>
<td>70</td>
</tr>
<tr>
<td>3.6</td>
<td><em>In Vivo</em> deletion of Wt1 has an effect on the GFP subpopulations</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>in the bone marrow</td>
<td></td>
</tr>
<tr>
<td>3.7</td>
<td><em>In Vivo</em> deletion of Wt1 has an effect on the GFP subpopulations in bone</td>
<td>72</td>
</tr>
<tr>
<td>3.8</td>
<td><em>In Vitro</em> Wt1 Deletion has no effect on bone marrow or bone</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>subpopulations cultured under normoxia (n) or hypoxia (h)</td>
<td></td>
</tr>
<tr>
<td>3.9</td>
<td><em>In Vitro</em> Wt1 Deletion has no effect on bone marrow subpopulations, however</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>hypoxia does</td>
<td></td>
</tr>
<tr>
<td>3.10</td>
<td><em>In Vitro</em> Wt1 Deletion has an effect on the bone GFP population</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>but not on subpopulations, however hypoxia does</td>
<td></td>
</tr>
<tr>
<td>3.11</td>
<td>Representative FACS plot summarising the 4 different quartiles described by</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Nakamura et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>3.12</td>
<td>The <em>in vivo</em> osteogenic distribution of Lin(^-)CD31(^-) marrow and</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>GFP cells</td>
<td></td>
</tr>
<tr>
<td>3.13</td>
<td>The <em>in vivo</em> osteogenic distribution of Lin CD31(^-) bone and</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>GFP cells</td>
<td></td>
</tr>
<tr>
<td>3.14</td>
<td>The <em>in vitro</em> osteogenic distribution of total marrow and GFP cells</td>
<td>84</td>
</tr>
<tr>
<td>3.15</td>
<td>The <em>in vitro</em> osteogenic distribution of total bone and GFP cells</td>
<td>85</td>
</tr>
</tbody>
</table>
Chapter 4

Figure 4.1. Preliminary data suggests non-haematopoietic GFP positive MSCs (Lin^CD31^-Triple^GFP^) are able to differentiate in vitro into 3 lineages based on morphology

Figure 4.2. The GFP^+ percentage of total marrow cells increases during adipogenesis

Figure 4.3. Wt1 mRNA expression levels increase during adipogenesis

Figure 4.4. Representative images for each CFU-F culture well

Figure 4.5. CFU-F Assay: Total number of colonies is reduced in Wt1 mutant

Figure 4.6. CFU-F Assay: Number of large colonies is reduced in Wt1 mutant

Figure 4.7. CFU-F Assay - Male: Area and diameter of colonies are reduced in Wt1 mutant

Figure 4.8. CFU-F Assay - Female: Area and diameter of colonies are reduced in Wt1 mutant

Figure 4.9. A representation of CFU-F wells from 4 mice

Figure 4.10. CFU-F: Activated CreER recombinase results in reduced marrow colony numbers

Figure 4.11. Lineage Tracing

Chapter 5

Figure 5.1. The breakdown of 45 differential gene expression changes between control marrow cells and mutant marrow cells

Figure 5.2. The breakdown of 8,714 differential gene expression changes between control bone cells and mutant bone cells

Figure 5.3. GO terms branching from anatomical structure development shown by GOrilla visualization tool

Figure 5.4. GO terms branching from developmental process shown by GOrilla visualization tool

Figure 5.5. Immune response GO terms shown by GOrilla visualization tool

Figure 5.6. Hypoxic response GO terms shown by GOrilla visualization tool

Figure 5.7. Bone remodelling GO terms shown by GOrilla visualization tool

Figure 5.8. RNA related GO terms shown by the GOrilla visualization tool to be down regulated in Cre^- bone cells

Figure 5.9. Bone homeostasis related GO terms shown by the GOrilla visualization tool to be up regulated in Cre^+ bone cells

Figure 5.10. Normalisation boxplot showing all the data compensated for against batch effects to allow for comparisons

Figure 5.11. Gene expression of Wt1 deletion mutant bone cells differs to the gene expression of WER control bone cells, CAGG-CreER™ Cre^-, and Cre^+ bone cells

Figure 5.12. Examples of genes not affected by CreER activation

Figure 5.13. Examples of genes affected by CreER activation

Figure 5.14. qRT-PCR validating the down regulation of genes associated with the collagen biosynthetic process GO term in Wt1 deletion mutant bone cells cultured in vitro
Figure 5.15. qRT-PCR validating the down regulation of genes associated with the cartilage development GO term in Wt1 deletion mutant bone cells cultured *in vitro* 132

Figure 5.16. qRT-PCR validating the down regulation of genes associated with the muscle tissue morphogenesis GO term in Wt1 deletion mutant bone cells cultured *in vitro* 133

Figure 5.17. qRT-PCR validating the down regulation of genes associated with the positive regulation of epithelial to mesenchymal transition GO term in Wt1 deletion mutant bone cells cultured *in vitro* 134

Figure 5.18. qRT-PCR validating the down regulation of genes associated with positive regulation of Wnt signalling GO term in Wt1 deletion mutant bone cells cultured *in vitro* 135

Figure 5.19. qRT-PCR validating the down regulation of genes associated with the angiogenesis regulation GO term in Wt1 deletion mutant bone cells cultured *in vitro* 136

Figure 5.20. qRT-PCR validating the down regulation of genes associated with response to hypoxia GO term in Wt1 deletion mutant bone cells cultured *in vitro* 137

Figure 5.21. qRT-PCR validating the up regulation of genes associated with inflammatory response GO term in Wt1 deletion mutant bone cells cultured *in vitro* 138

Figure 5.22. Lipid metabolism GO terms shown by the GOrilla visualization tool to be down regulated in mutant GFP<sup>+</sup> bone cells 142

Figure 5.23. RNA processing GO terms shown by the GOrilla visualization tool to be down regulated in mutant GFP<sup>+</sup> bone cells 143

Figure 5.24. RNA processing GO terms shown by the GOrilla visualization tool to be down regulated in mutant GFP<sup>+</sup> bone cells 144

Figure 5.25. Apoptosis related GO terms shown by the GOrilla visualization tool to be down regulated in mutant GFP<sup>+</sup> bone cells 144

Figure 5.26. Loss of Wt1 causes Cxcl1 and Cxcl10 expression to significantly increase in cultured bone marrow 146

**Chapter 6**

Figure 6.1. The mean percentage (±SEM) of total cells which are GFP<sup>+</sup> decreases as development progresses from e11.5 to e16.5 156

Figure 6.2. GFP and Wt1 DAB immunohistochemistry on e11.5 sections 157

Figure 6.3. GFP and Wt1 immunofluorescence on e11.5 sections 158

Figure 6.4. GFP and Wt1 DAB immunohistochemistry on e14.5 sections 159

Figure 6.5. GFP and Wt1 immunofluorescence on e14.5 sections 160

Figure 6.6. Wt1 DAB immunohistochemistry on e15.5 sections 161

Figure 6.7. GFP and Wt1 immunofluorescence on e15.5 sections 162

Figure 6.8. Wt1 DAB immunohistochemistry on e16.5 sections 163

Figure 6.9. GFP DAB immunohistochemistry on e16.5 sections 164

Figure 6.10. GFP and Wt1 immunofluorescence on e16.5 sections 165
Figure 6.11. The mean percentage of total hindlimb cells which are non-haematopoietic (i.e. Lin-CD31-) increases following the deletion of Wt1

Figure 6.12. The mean percentage of total hindlimb cells which are non-haematopoietic MSCs (i.e. Lin-CD31-Triple+) decreases following the deletion of Wt1

Figure 6.13. Hematoxylin and eosin staining of e18.5 hindlimb shows no obvious difference between control mice and Wt1 deletion mice

Figure 6.14. Limb cells during development mainly express markers for osteoblast progenitors

Figure 6.15. In utero deletion of Wt1 seems to have little effect on the mean percentage of cells expressing osteoblast progenitor markers

Figure 6.16. Lineage tracing in the leg induced at e14.5 and analysed at e18.5

Figure 6.17. Lineage tracing induced at e14.5 and analysed at 10 days

Figure 6.18. Lineage tracing induced at e14.5 and analysed at 3 weeks

Figure 6.19. Lineage tracing induced at e14.5 and analysed at e18.5

Figure 6.20. Lineage tracing induced at e14.5 and analysed at e18.5

Figure 6.21. Lineage tracing induced at e14.5 and analysed at e18.5

Figure 6.22. Lineage tracing induced at e14.5 and analysed at 10 days

Figure 6.23. Lineage tracing induced at e14.5 and analysed at 10 days
## List of Tables

### Chapter 2

| Table 2.1 | Sample Processing Conditions and Timings | 50 |
| Table 2.2 | Antibody Information | 53 |
| Table 2.3 | Cycling Conditions | 56 |
| Table 2.4 | Primer Information for Gel Electrophoresis of DNA samples | 56 |
| Table 2.5 | Primer and probe information for each gene used in qRT-PCR | 58 |
| Table 2.6 | Cycling conditions for qRT-PCR | 58 |
| Table 2.7 | Cycling conditions for qRT-PCR Custom Plates | 60 |
| Table 2.8 | FACS Antibody dilution and wavelength information | 61 |

### Chapter 4

| Table 4.1 | Average number (±SEM) of colonies from marrow cells treated with tamoxifen with and without *loxP* sites | 105 |
| Table 4.2 | *In vivo* lineage tracing | 106 |

### Chapter 5

<p>| Table 5.1 | Genes increased in Wt1 deletion mutant marrow cells cultured under normoxia | 114 |
| Table 5.2 | Genes increased in Wt1 deletion mutant marrow cells cultured under hypoxia | 114 |
| Table 5.3 | Genes decreased in Wt1 deletion mutant marrow cells cultured under hypoxia | 114 |
| Table 5.4 | Enriched GOrilla GO terms of genes down regulated in Wt1 deletion mutant bone | 114 |
| Table 5.5 | Enriched GOrilla GO terms of genes down regulated in Wt1 deletion mutant bone | 115 |
| Table 5.6 | Enriched GOrilla GO terms of genes down regulated in Wt1 deletion mutant bone | 116 |
| Table 5.7 | Enriched DAVID GO terms of genes down regulated in Wt1 deletion mutant bone | 117 |
| Table 5.8 | Enriched GOrilla GO terms of genes up regulated in Wt1 deletion mutant bone | 117 |
| Table 5.9 | Enriched DAVID GO terms of genes up regulated in Wt1 deletion mutant bone | 119 |
| Table 5.10 | Enriched GOrilla GO terms of genes up regulated in bone cells cultured under hypoxic conditions | 119 |
| Table 5.11 | Enriched GOrilla GO terms of genes down regulated in bone cells cultured under hypoxic conditions | 120 |
| Table 5.12 | Enriched DAVID GO terms of genes down regulated in bone cells cultured under hypoxic conditions | 120 |</p>
<table>
<thead>
<tr>
<th>Table 5.13.</th>
<th>Genes which are significantly differentially expressed in Cre⁺ bone cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 5.14.</td>
<td>Enriched GOrilla GO terms of genes down regulated in Cre⁺ bone cells cultured under hypoxic conditions</td>
</tr>
<tr>
<td>Table 5.15.</td>
<td>Enriched GOrilla GO terms of genes up regulated in Cre⁺ bone cells cultured under hypoxic conditions</td>
</tr>
<tr>
<td>Table 5.16.</td>
<td>Enriched GOrilla GO terms of genes up regulated in Cre⁺ bone cells cultured under hypoxic conditions</td>
</tr>
<tr>
<td>Table 5.17.</td>
<td>Genes associated with the bone homeostasis GO terms enriched in the CAGG-CreER positive;Wt1⁺/⁺ bone cells</td>
</tr>
<tr>
<td>Table 5.18.</td>
<td>Enriched GOrilla GO terms of genes down regulated in GFP⁺ Wt1 mutant bone cells cultured under hypoxic conditions</td>
</tr>
<tr>
<td>Table 5.19.</td>
<td>Enriched GOrilla GO terms of genes down regulated in GFP⁺ Wt1 mutant bone cells cultured under hypoxic conditions</td>
</tr>
<tr>
<td>Table 5.20.</td>
<td>Enriched GOrilla GO terms of genes up regulated in GFP⁺ Wt1 mutant bone cells cultured under hypoxic conditions</td>
</tr>
<tr>
<td>Table 5.21.</td>
<td>Enriched GOrilla GO terms of genes down regulated in GFP⁺ Wt1 mutant bone cells cultured under hypoxic conditions</td>
</tr>
<tr>
<td>Table 5.22.</td>
<td>Genes which are significantly differentially expressed in GFP⁺ bone cells</td>
</tr>
<tr>
<td>Table 5.23.</td>
<td>Enriched GOrilla GO terms of genes up regulated in GFP⁺ bone cells cultured under hypoxic conditions</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-MSC</td>
<td>bone marrow derived mesenchymal stem cell</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFU-F</td>
<td>colony forming unit-fibroblast</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>D-MEM</td>
<td>Dulbecco’s-minimal Eagle’s medium</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>Dapi</td>
<td>4’,6-diamidino-2-phenyindole</td>
</tr>
<tr>
<td>dH2O</td>
<td>deionised water</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>e</td>
<td>embryonic stage</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>ERT</td>
<td>oestrogen receptor tamoxifen inducible</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescent activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>HSC</td>
<td>haematopoietic stem cell</td>
</tr>
<tr>
<td>Ig</td>
<td>immunooglobin</td>
</tr>
<tr>
<td>K</td>
<td>lysine</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LSC</td>
<td>liver stem cell</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MET</td>
<td>mesenchymal to epithelial transition</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>MPC</td>
<td>mesenchymal progenitor cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µm</td>
<td>micrometre</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative realtime polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>S</td>
<td>serine</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>T</td>
<td>threonine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-EDTA boric acid buffer</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet radiation</td>
</tr>
<tr>
<td>WER</td>
<td>CAGG-CreER&lt;sup&gt;T2;WT1&lt;sub&gt;loxP/loxP&lt;/sub&gt;&lt;/sup&gt;</td>
</tr>
<tr>
<td>WGER</td>
<td>CAGG-CreER&lt;sup&gt;T2;WT1&lt;sub&gt;loxP/GFP&lt;/sub&gt;&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms’ Tumour 1</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1 Bone Formation

The bones of the body vary in length, size, and shape; the cranial bones, for example, differ greatly in physical shape to the tibia. However, they are all made up of the same cell types: osteoblasts, osteoclasts, osteocytes, vascular cells, and sometimes marrow cells (Caplan, 1988).

There are 2 main types of bone formation: endochondral (indirect) and intramembranous (direct) ossification. Intramembranous ossification is the direct laying down of osteoblasts into the primitive connective tissue (Karaplis, 2008). This type of bone formations occurs in the absence of cartilage, with mesenchymal cells directly differentiating into osteoblasts (Karaplis, 2008). Alternatively, endochondral bone formation occurs when the cartilage inside the metaphysis of long bones calcifies. The metaphysis is the area of long bone between the epiphysis (the bone end) and the narrow diaphysis middle. This becomes a mineralised core, providing a surface on which osteoblasts form new bone (Riminucci et al., 1998). Perichondral bone formation also occurs during initial long bone development (Riminucci et al., 1998). It takes place outside of the cartilage, before mineralisation has occurred, to produce bone collars around the cartilage and is associated with the periosteal layer (Riminucci et al., 1998).

Mesenchymal cells undergo division and maturation resulting in hypertrophic cartilage, which is then eroded by blood vessels, osteoclasts, and haematopoietic marrow to be replaced by trabecular bone below the growth plates at the bone ends i.e. epiphyseal cartilage (Caplan, 1991; Zelzer and Olsen, 2005). Embryonic bone development takes place when a core of prechondrogenic cells is surrounded by stacked osteogenic progenitor cells, all of which are avascular. This central core and surrounding stacked cells differentiate into cartilage to give a cartilage rod or ‘model’. The stacked cells then give rise to osteoblasts (Pechak et al., 1986a; 1986b). Osteoblasts are located in close proximity to blood vessels with the “back” of the cell toward the capillary and the “front” of the cell secreting osteoid; a Collagen Type I rich premineral layer (Pechak et al., 1986a; 1986b). Caplan (1987)
reviews how the osteoid is laid down in layers before being mineralised after which a layer of vasculature is formed, and topped by another layer of mineralised osteoid. This alternate layering continues until there are around 10-15 layers which restricts the expansion of the cartilaginous core (Caplan, 1987; Caplan, 1988). Vasculature is the dominant factor for the position and survival of the osteoblast cells, unlike chondrogenesis which actively inhibits vascularisation (Caplan, 1987; Caplan 1988). For this reason bone does not replace cartilage, but cartilage is invaded by vascular cells to give the resulting marrow cavity (Pechak et al., 1986b), and bone forms independent of the cartilage (Caplan, 1987; Caplan, 1988). In mice, bone development begins with mesenchyme condensing at embryonic day 9.5 (e9.5). By e12 the commitment of mesenchymal cells to the osteogenic lineage occurs, followed by commitment to the chondrogenic lineage on day 13, finishing with vasculature and marrow invasion of the cartilage occurring on days 16-17 of development (Caplan, 1988; Zelzer and Olsen, 2005). The process of bone formation is outlined schematically in Figure 1.1.

![Figure 1.1](image_url)

**Figure 1.1 shows bone formation in a mouse tibia.** The mesenchyme condenses and differentiates into chondrocytes to form avascular cartilage. At e14 (ED14) the central chondrocytes become hypertrophic and are then invaded by vasculature as the bone collar forms at the perichondrium. The marrow cavity then expands by eroding the cartilage in the growth plates of the epiphyses. As the hypertrophic cartilage is removed, the area below the growth plate epiphyseal cartilage is replaced by trabecular bone (modified from Horton, 1990; Zelzer and Olsen, 2005).

Studying the osteoblast lineage pathway has shown that osteocytes are the terminally differentiated cell of the osteoblast lineage (Caplan, 1991; Noble, 2008). Together
osteoblasts and osteocytes are responsible for bone turnover, repair, and mineral homeostasis (Noble, 2008). Osteocytes, the most abundant cell type in the bone (Mullender et al., 1996), are non-proliferative cells which are found in the osteoid before mineralisation, and in the bone matrix after mineralisation (Noble, 2008). They have a star shaped morphology due to their dendritic processes which allow them to form a signalling network through the bone (Noble, 2008).

The structure of bone is such that it gives maximum strength with minimum mass and is maintained by constant remodelling consisting of bone synthesis and resorption (Boyle et al., 2003). A key player in bone remodelling is the osteoclast cell responsible for bone resorption (Teitelbaum, 2000; Boyle et al., 2003). Osteoclasts are tissue-specific macrophage polykaryons originating from monocyte/macrophage progenitors near the surface of the bone (Teitelbaum, 2000; Boyle et al., 2003). They are activated by signals, such as RANK ligand (RANKL) also known as osteoprotegerin ligand (OPGL), which leads to bone remodelling (Lacey et al., 1998; Boyle et al., 2003). When the balance between osteoclasts and osteoblasts is disrupted, consequently altering the bone resorption and formation balance, skeletal diseases such as osteoporosis and rheumatoid arthritis can result (Kong et al., 1999; Rodan and Martin, 2000).

Embryonic bone development and bone repair are two different events (Caplan, 1987). Some of the bioactive factors involved in bone repair are not involved in the differentiation of mesenchymal cells in bone formation (Caplan, 1987). It is well known that bone is highly capable of repairing any discontinuities or damage, and factors have been found in the bone to promote repair (Caplan, 1991). When a bone breaks, the broken bone itself provides autocrine factors, whilst responding cells provide paracrine signals including various hormones and growth factors e.g. PDGF and heparin (Caplan, 1987). The autocrine factors include MSC chemoattractants as well as biofactors which cause MSC differentiation into chondrocytes. Historically one such biofactor was Chondrogenic Stimulating Activity (CSA) (Caplan, 1987). An assay was used to purify bioactive molecules from the demineralised bone matrix and identified CSA as a 31kDa protein which is now known to be a heterodimer of
bone morphogenetic proteins (BMPs) (Syftestad and Caplan, 1984; Syftestad et al., 1985). CSA was previously shown to promote chondrogenesis but was not detected prior to initial osteogenesis as it is produced by the osteoblasts found in the newly formed bone cells (Syftestad and Caplan, 1984; Syftestad et al., 1985; Caplan, 1987). Canalis et al. (2003) reviewed the important role that these TGF-β superfamily members, the BMPs, play. They are needed for MSC differentiation along the osteoblastic lineage, plus osteoblast maturation and survival; are required directly and indirectly for osteoclastogenesis; and can induce endochondral ossification, chondrogenesis, and chondrocyte maturation (Canalis et al., 2003).

As well as BMPs, there are various other local factors and systemic hormones which, singularly or together, modify and regulate homeostasis in the bone microenvironment. Examples of these are Insulin-like growth factors (IGF), Runx-related transcription factor-2 (Runx-2), and Osterix. IGFs in the bone are regulated by their binding proteins (IGFBPs) which are produced by osteoblasts (Hwa et al., 1999). Runx-2 is vital for undifferentiated cells to follow the osteoblast lineage pathway (Banerjee et al., 2001), as well as regulating chondrocyte maturation, making it essential for ossification (Komori et al., 1997). Runx-2 and BMP-2 interact together to mediate osteoblast gene transcription (Banerjee et al., 2001). Another important transcription factor is Osterix which is expressed by osteoblasts and required for both endochondral and intramembranous bone ossification (Nakashima et al., 2002). It is found downstream in the Runx-2 pathway and requires Runx-2 for its expression (Nakashima et al., 2002).

When a bone breaks, cartilage forms to link the two broken sections acting as a scaffold and allowing osteogenesis around it to result in continuous bone (Caplan, 1987). As described during development, the cartilage formed at the break is targeted by the vasculature and reforms the marrow cavity (Caplan, 1987). This invaded cartilage, however, is different to the cartilage found at the articular surface, the surface at the joint of two bones, which is never replaced by marrow or invaded by vasculature (Caplan, 1987).
1.2 Bone Marrow Overview

Post-natal bone marrow is an organ of two halves; the haematopoietic cells, including the haematopoietic stem cells (HSCs), and the non-haematopoietic cells (Bianco et al., 2001). The non-haematopoietic cells are also known as the bone marrow stroma, or associated supporting stroma, and this is where the mesenchymal stem cell resides (Bianco et al., 2001). The bone marrow is the only known organ where two separate stem cells, MSCs and HSCs, with distinct lineage pathways are located and found to interact together to coexist (Bianco et al., 2001).

1.2.1 Bone marrow stroma is a heterogeneous population

Friedenstein et al. (1966) used intraperitoneal transplantation of diffusion chambers containing either bone marrow fragments, or bone marrow cells, to characterise bone marrow stroma as a heterogeneous population and show that reticular cells are formed as well as bone (provided the correct cell density is transplanted). Owen (1988) further reviewed the utilisation of in vitro culture studies, in vivo ectopic marrow transplantation e.g. subcutaneously, and in vivo diffusion chambers to characterise the heterogeneous nature of the bone marrow stromal cells. When bone marrow cells were plated out at low densities the spindle-shaped stromal cells adhered to the plastic culture dishes, whereas the haematopoietic cells were nonadherent and were easily washed from the dish (Friedenstein et al., 1970; Bianco et al., 2001). With further culture these adherent stromal cells formed colonies originating from a single cell; the colony forming unit-fibroblast (CFU-F) (Friedenstein et al., 1970). CFU-F assays are frequently used to characterise stromal marrow cells. The variation seen in colony size and morphology indicates the heterogeneity of these cells (Bianco et al., 2001). Cells from multiple colonies transplanted into host animals, either in porous sponges or diffusion chambers, have resulted in ossicle formation with supporting bone marrow stromal cells, adipocytes, and cartilage (Ashton et al., 1980; Friedenstein et al., 1982). However, when subcutaneous transplantation was carried out using a strain of cells which originated from a single CFU-F, i.e. cells of a single colony, only 58.8% of transplants had the
ability to form bone, compared with 100% bone formation in the multi-strain implantations (i.e. strains formed from multiple CFU-Fs) (Kuznetsov et al., 1997). These results highlight the “stemness” of the original CFU-F cell, but also that the osteogenic capability varies between CFU-Fs (Kuznetsov et al., 1997). Bianco et al. (2001) discussed the shared aspects of bone marrow stromal cells and fibroblasts, such as expression of α-smooth muscle actin (α-SMA) and matrix proteins, as well as expressing endoglin similar to endothelial cells. However, on the whole the bone marrow stromal cells, encompassing the MSCs, are a challenging set of cells to characterise completely due to the way they constantly change and respond to their microenvironment (Bianco et al., 2001).

1.2.2 Vascularisation is vital for bone marrow formation

Primitive marrow is formed when the chondrocyte core is invaded by vasculature to form the marrow cavity (Pechak et al., 1986b). It is unsurprising, therefore, that the relationship between marrow stromal cells and vasculature is also present in the adult marrow (Bianco et al., 2001). This vascular network is lined with endothelial cells plus another layer of cells below this called pericytes (Andreeva et al., 1998). This subendothelial layer of pericytes is located in every region of the vascular bed; from large and medium blood vessels down to capillaries and microcirculatory vessels (Andreeva et al., 1998). Capillary pericytes have small bodies with an intricate and reticular-like shape defined by long processes which provide a cell-cell contact network. In the venous network, the pericyte’s small body becomes elongated and the processes flatten out to become wider (Andreeva et al., 1998; Bianco et al., 2001). Reticular cells in the marrow are a specialised type of pericyte whose cellular properties differ depending on their location along the marrow vasculature (Bianco et al., 2001). Reticular cells found in the venous capillary adventitia, or outer connective tissue of the vessel, are able to accumulate lipids and become adipocytes making them a unique specialised pericyte (Bianco et al., 1988). The conversion of these pericytes into adipocytes provides the vessel in question with an enforced basement membrane minimising its permeability and sometimes causing the lumen to collapse, reversibly removing the vessel from circulation (Bianco et al., 1988). A
normal pericyte layer generally ensures a stable and supported vessel, whereas loss of pericytes is linked with vessel regression (Benjamin et al., 1998). Studies performed in developing retinal vasculature show that the formation of the pericyte layer is delayed and lags behind the developing blood vessels by up to a week (Benjamin et al., 1998). Pericyte coating only becomes complete once adulthood is reached when vessels are coated from the arterioles to the venules following a particular temporal pathway (Benjamin et al., 1998). The delay between vessel formation and pericyte coating allows a window of plasticity in which the vasculature is able to be altered and remodelled depending on the requirements of the tissue (Benjamin et al., 1998). The reversible regulation of the marrow vasculature is therefore controlled by the marrow itself, specifically adipose converting pericytes, which can also fill any inactive empty areas of the marrow cavity with fat (Bianco et al., 1988; Bianco et al., 2001).

*In vivo* α-SMA expression in non-vascular marrow stromal cells is linked with marrow growth and angiogenesis, a process which requires parallel growth between pericytes and endothelial cells (Bianco et al., 2001). Galmiche et al. (1993) showed that in human adult bone marrow α-SMA is expressed by smooth muscle cells in the arterioles, myoid cells, and pericytes. However, α-SMA expressing stromal cells are more similar to pericytes than smooth muscle cells when observed in culture (Galmiche et al., 1993). Their findings showed that smooth muscle-like stromal cells can be derived from marrow mesenchymal cells *in vitro*, and *in vivo* can produce in myoid cells and pericytes, depending on location and need (Galmiche et al., 1993).

Bone formation by osteoblasts, endothelial cell growth, and formation of the outer lining of pericytes all occur at specific time points relative to each other (Bianco et al., 2001). This can be observed best at the metaphyseal growth plates, found at the end of the long bone structure just before the epiphyseal growth plates at the bone terminus (Hunter et al., 1991). The epiphyseal cartilage growth plate is vital for endochondral bone formation and is made up of longitudinally aligned chondrocytes (Hunter et al., 1991). Later in development this cartilage is mineralised and invaded by metaphyseal vasculature called capillary sprouts (Hunter et al., 1991). In rats, the
initial 200 microns of the metaphysis growth plate is made up of growing endothelial tubes which lack a basement membrane and pericyte layer (Hunter et al., 1991). At around 300 microns from the tip of these endothelial sprouts the microvasculature is more mature and now has a basement membrane and covering of pericytes (Hunter et al., 1991). These vessels give rise to the growing metaphyseal sprouts; pericytes and osteoblasts are now present as bone formation is reliant on angiogenesis and marrow vascularisation (Hunter et al., 1991). Interestingly, many properties of the marrow pericytes closely mimic marrow stromal cells, which leads us to wonder whether pericytes are potentially marrow progenitor cells (Shepro and Morel, 1993). Díaz-Flores et al. (1991a) reviewed the mesenchymal potential and pluripotency of these cells and discussed pericytes as a source of undifferentiated mesenchymal progenitors used for repair. Pericytes are thought to have various sources of origin and some studies suggest that they are capable of forming cartilage and bone in vivo (Díaz-Flores et al., 1991b; 1992). Studies provide evidence that pericytes can arise from bone marrow cells during angiogenesis (Kokovay et al., 2006), from endothelial cells (DeRuiter et al., 1997), from embryonic stem cell derived Flk1-expressing cells (which also give rise to endothelial cells) (Yamashita et al., 2000), and from the proliferation and migration of other pericytes found downstream of a site of vascularisation, which can be either independent or dependent on Platelet-derived growth factor – B (PDGF-B) (Hellström et al., 1999). This means that pericytes in the bone marrow may be of different origins adding to the heterogeneity.

1.2.3 Bone marrow stromal cells have conventional and unconventional plasticity

It seems that marrow stromal cells have a certain amount of plasticity. To add to the earlier example of pericytes becoming adipocytes, marrow adipocytes themselves can be pushed toward osteogenesis and form bone in vivo (Bennett et al., 1991). Fibrous-osteogenic tissue was observed when adipocytic colonies were implanted in diffusion chambers, however it is not known whether osteogenesis is limited by the stage of adipocytic differentiation; it may be limited to preadipocytic cells, or capable by mature adipocytes (Bennett et al., 1991). As well as this, chondrocytes
which have reached terminal differentiation in the marrow, i.e. hypertrophic chondrocytes, are capable of dedifferentiating \textit{in vitro} and following an osteogenic lineage pathway (Gentili \textit{et al.}, 1993). Gentili \textit{et al.} (1993) hypothesised that cross-talk occurs between hypertrophic chondrocytes and preosteoblasts to trigger the bone formation. There is also evidence of this occurring \textit{in vivo} where chondrocytes in the early stage of hypertrophy start to express osteogenic traits (Riminucci \textit{et al.}, 1998). Differentiation into these cells (e.g. osteoblasts, chondrocytes, adipocytes, and reticular cells) was always thought to be a terminal, irreversible process as is the case with haematopoietic cells but this appears not to be true. Red blood cells are not capable of differentiating into white blood cells at the last minute if required, whereas the bone marrow stromal cells seem to have the option of dedifferentiation (Bianco \textit{et al.}, 2001). This plasticity is relatively conventional in that the switches in differentiation are occurring \textit{within} the same organ system, e.g. chondrocytes into osteocytes, within the skeletal tissue. However, more unconventional plasticity has been documented where progenitors found in the bone marrow have given rise to completely unrelated tissues (Bianco \textit{et al.}, 2001). There are several examples of this:

i) Transplanted bone marrow cells can give rise to neural progenitors which express neuronal antigens in the adult brain (Mezey \textit{et al.}, 2000).

ii) Bone marrow cells transplanted into mice with lethal liver disease give rise to hepatocyte progenitors which have rescued the mice (Lagasse \textit{et al.}, 2000).

iii) Bone marrow stromal cells injected in the lateral ventricle can migrate through the forebrain and cerebellum, can differentiate into astrocytes, and can populate various neuron rich areas in the brain, including the brain stem, which suggests these cells are differentiating into neurons (Kopen \textit{et al.}, 1999).

iv) Transplanted bone marrow cells migrate to areas of muscle damage, differentiate into myocyte progenitors, and can result in terminally differentiated muscle fibres (Ferrari \textit{et al.}, 1998).
1.3 Mesenchymal Stem/Progenitor Cell definition

Mesenchymal stem cells, or mesenchymal stromal cells (MSCs), are commonly described as spindle-shaped multipotent cells able to self-replicate and generate progenitors that produce a variety of skeletal tissues: bone, cartilage, marrow stroma, fat, ligament, tendon, and connective tissue. This is known as the mesengenic process and is outlined in Figure 1.2. MSCs are able to divide indefinitely. The progenitors, however, are directed down different and specific routes of differentiation regulated by both paracrine and autocrine effectors until they become committed to a specific cell type (Caplan, 1986; Caplan, 1987; Caplan, 1988). Caplan (1991) highlights the importance of mesenchymal stem cells and their role in bone and cartilage formation in adult bone homeostasis. In development, the embryo is divided into 3 layers: ectoderm, mesoderm, and endoderm. The middle mesodermal layer gives rise to the mesenchymal elements including the skeletal tissues (Caplan, 1991). The word ‘mesenchyme’ comes from the Greek for “middle” and “infusion” which describes how these cells move within and populate the area between the ectoderm and endoderm (Caplan, 1991). The migratory and specific differentiation properties of these cells make them well suited for the repair of damaged tissues in adults, such as wounded bone and muscle (Caplan, 1991). Caplan (1991) goes on to review experiments determining conditions for chondrocyte and osteoblast differentiation, concluding that culture conditions differ for the two lineages despite being from a shared origin. This highlights the fact that progenitors become committed to particular lineages, and this commitment appears to follow a time scale as chondrogenesis follows a genetically programmed pathway (Caplan, 1984). This genetic regulation has now been shown to work through several clock genes, including Per1, Bmal1, and Clock, that are expressed by chondrocytes (Takarada et al., 2012). A mechanism involving the protein products of these genes and the master regulator gene, Indian hedgehog (Ihh), means chondrogenesis in the growth plate is under oscillatory regulation (Takarada et al., 2012).
Mesenchymal stem cells are still an ill-defined population due to their heterogeneity. However, the Tissue Stem Cell Committee of the International Society for Cellular Therapy provided three criteria as a minimum definition for human MSCs (Dominici et al., 2006):

1) MSCs must adhere to plastic.
2) MSCs must express CD105, CD73, and CD90 surface markers, and lack expression of the following haematopoietic surface markers: CD45, CD34, CD14 or CD11b, CD19 or CD79α, and HLA class II.
3) MSCs must differentiate into osteoblasts, chondroblasts, and adipocytes in vitro.

Dominici et al. (2006) emphasised that these criteria are proposed as minimum requirements and that investigators should use as many positive and negative surface markers as is suitable for the research questions being addressed. They also highlight that these criteria are specific for human MSCs. Plastic adherence and
differentiation into the three lineages are also properties of murine MSCs (Tropel et al., 2004), however the surface cell marker profile in mice is not well characterised.

MSCs are considered a heterogeneous population which is highlighted by the surface markers expressed by these cells. Analysis of the human MSC gene expression profile showed that many of the cell surface antigens expressed by the MSCs are also expressed by and characteristic for other cell types (Silva et al., 2003). Most studies investigating MSCs have been carried out on human cells. It is much more difficult to isolate murine MSCs from the bone marrow than human, and consequently there is a lot less information on murine MSCs (Sun et al., 2003; Peister et al., 2004). This lack of information makes isolating murine MSCs very uncertain and highlights a need for specific markers and methods for their isolation.

Baddoo et al. (2003) showed that murine MSCs could be isolated from plastic adherent cultured bone marrow cells where haematopoietic cells were negatively selected for using CD11b, CD34, and CD45 markers. The remaining immunodepleted population expressed the following cell surface markers: Sca-1, CD9, CD29, CD44, CD81, and CD106, and didn’t express CD48, CD90, CD117 (c-kit), CD135, CD31, or the transcription factor Oct-4 (Baddoo et al., 2003). These immunodepleted cells also had the ability to differentiate into osteoblasts, chondrocytes, and adipocytes in vitro (Baddoo et al., 2003).

Platelet-derived growth factor receptor α (PDGFR-α) and Stem cell antigen 1 (Sca-1) are two positive markers which have also been used to identify a murine MSC population from marrow and bone (Morikawa et al., 2009). Unlike other studies where marrow cells are flushed from the bone, this study combines marrow with bone which has been crushed with a collagenase digest (Morikawa et al., 2009). This extra bone digest step was included as MSCs have been located near the endosteum of the bone, in the bone lining endosteal region, where they interact with bone lining osteoblasts (Muguruma et al., 2006). Haematopoietic cells were identified and negatively sorted using lineage-specific antibodies including CD45 and Ter-119 (Morikawa et al., 2009). The PDGFRα⁺Sca-1⁺ cells were found located in the perivascular space near the inner cortical bone surface (Morikawa et al., 2009).
They have self-renewal properties as well as the capacity for osteogenic and chondrogenic differentiation. However these cells were also capable of angiogenic differentiation and expressed angiopoietin-1 (Ang-1) suggesting they are both mesenchymal and perivascular progenitors (Morikawa et al., 2009). Cultured PDGFRα-Sca-1+ cells expressed the following conventional MSC markers: CD29, CD44, CD49e, CD90, and CD105, which are markers originally used for cultured human MSCs (Morikawa et al., 2009).

A subset of bone marrow derived MSCs has also been identified by the expression of nestin, where cells express GFP driven by the nestin promoter (Méndez-Ferrer et al., 2010). These non-haematopoietic nestin positive cells had upregulated differentiation genes for osteogenesis, adipogenesis, and chondrogenesis, as well as forming osteoblasts, osteocytes, and chondrocytes in vivo, and all three lineages in vitro (Méndez-Ferrer et al., 2010). These nestin+ cells posses self-renewal properties and were also found close to HSCs in the haematopoietic stem cell niche, and were required for HSC maintenance and survival (Méndez-Ferrer et al., 2010).

As stated earlier, Dominici et al. (2006) suggest that to be a human MSC the cell must express CD105, CD73, and CD90. For this study we have chosen to identify our MSC population using the cell surface markers CD105, CD73, and CD29. There are studies to support the expression of CD105 and CD73 in murine MSCs as well as human (Sun et al., 2003; Eliopoulos et al., 2005; Breitbach et al., 2007; Morikawa et al., 2009). However, there are conflicting reports on the expression of CD90 in murine cells, some suggesting it is negative (Baddoo et al., 2003) and some positive (Morikawa et al., 2009). Peister et al. (2004) looked at CD90 expression in the adult bone marrow derived MSCs of four different mouse strains and showed them all to be negative for CD90, but showed positive expression in human MSCs. So this marker has been replaced by CD29, the expression of which has been verified in murine MSCs (Baddoo et al., 2003; Sun et al., 2003; Morikawa et al., 2009).

Marrow (Ohgushi et al., 1989a; 1989b), adipose tissue (Zuk et al., 2002), periosteum (O’Driscoll and Salter, 1984), bone (Guo et al., 2006), muscle connective tissue (Young et al., 1995), and fetal chorionic villi of the placenta (Igura et al., 2004), are
some of the sites where MSCs can be found, with adult liver also containing a population of MSC-like cells; liver stem cells (LSC) (Herrera et al., 2006).

MSCs in the Marrow

The bone marrow is the main source of MSCs. However, they make up a very small proportion of the total number of marrow cells, between 0.01% and 0.001% (Pittenger et al., 1999). Bone marrow contains MSCs which can undergo chondrogenic, osteogenic, and adipogenic differentiation to form cartilage, bone, and fat respectively (Caplan, 1991; Pittenger et al., 1999). Bone marrow derived MSCs (BM-MSCs) placed inside a diffusion chamber and implanted into the peritoneal cavity of a nude mouse rapidly proliferate and differentiate into cartilage in the centre of the chamber, and bone at the edges close to the host’s vasculature (Bab et al., 1988). Porous ceramic blocks soaked in a bone marrow cell suspension were inserted into the break site of a broken femur resulting in significant osteogenesis, some chondrogenesis, and a union of the two bone sections (Ohgushi et al., 1989a). Inserting the ceramic alone did not promote bone healing suggesting that the bone repair is due to the marrow cell solution (Ohgushi et al., 1989a). Osteogenesis was also observed when marrow cells with ceramics were implanted subcutaneously and intramuscularly (Ohgushi et al., 1989b).

The morphology of cultured MSCs derived from the bone marrow has been described as a relatively homogeneous population of spindle-shaped colonies by some studies (Pittenger et al., 1999), but others have found colonies derived from single cells to be more morphologically heterogeneous (Bruder et al., 1997). These colonies have been found to contain at least 2 cell types; small spindle shaped cells and larger cuboidal, flattened cells (Bruder et al., 1997). There have also been differences noted in proliferation rates, with small cells undergoing rapid proliferation and the larger cells renewing slower (Colter et al., 2000). A third population of very small and rapidly proliferating cells has been identified and thought to be the earliest progenitors with the highest potential for multilineage differentiation (Colter et al., 2001).
MSCs in Adipose Tissue

As previously discussed, bone marrow is derived from the mesenchyme, as is adipose tissue. There is a population of fibroblast-like cells within the adipose stromal compartment called processed lipoaspirate cells (PLA). Zuk et al. (2001) identified these cells and highlighted their similarities to MSCs. They showed that PLA cells are capable of osteogenic, adipogenic, myogenic, and chondrogenic differentiation in vitro (Zuk et al., 2001). The PLA cells expressed a number of CD marker antigens also expressed by MSCs, including CD29, CD44, CD71, CD90, CD105, SH3, and were negative for CD31, CD34, and CD45 (Zuk et al., 2002). However, there were differences in the expression of CD49d and CD106. There were also differences in certain gene expressions, for example, a lack of dlx5 and BMP-2 expression in PLA cells compared to MSCs (Zuk et al., 2002). Interestingly, some cells within the PLA population were able to give rise to clonal colonies containing all three lineages (osteoblast, adipocyte, chondrocyte), verified by quantitative PCR (Zuk et al., 2002). These cells are adipose-derived stem cells and they are responsible for the multi-lineage properties observed in the PLA cell population (Zuk et al., 2002).

MSCs in other sites:

Periosteum

The periosteum is a layer of cells located on the outer layer of long bone which responds to injury by increasing this cellular layer and producing woven bone (O’Driscoll and Salter, 1984). These periosteval cells were also able to differentiate into chondrocytes upon transplantation into an articular cartilage defect in the knee joint (O’Driscoll and Salter, 1984).

Bone

MSCs have been found in bone fragments which have been crushed and digested with a collagen enzyme. These cells were then able to differentiate into the 3 main mesenchymal lineages; osteocytes, chondrocytes, and adipocytes (Guo et al., 2006).
Connective Tissue
MSCs were isolated from the connective tissues of 26 separate organ tissues, including trachea, lungs, heart, kidney, liver, oesophagus, bone, cartilage, and vasculature. For all 26 cases they were able to differentiate into 4 different cells derived of the MSC lineage; cartilage, bone, adipocytes, and skeletal muscle (Young et al., 1995).

Placenta
Placenta-derived mesenchymal progenitor cells are located in the human fetal chorionic villi (Igura et al., 2004). These cells express the required MSC surface markers and were also able to differentiate into osteocytes, chondrocytes, and adipocytes.

Stem Cells in Adult Liver
Hepatocytes, obtained from digested liver tissue and then cultured in vitro, contain a population of self-renewing adherent cells, called Liver stem cells (LSC) (Herrera et al., 2006). LSCs express the MSC markers CD29, CD73, CD44, CD90, and lack HSC markers CD34, CD45, CD117, and CD133 (Herrera et al., 2006). They also express liver-specific proteins including albumin and α-fetoprotein, with a small population of cells also expressing cytokeratin-8 and cytokeratin-18 (Herrera et al., 2006). Although LSCs are capable of differentiation into hepatocytes, osteoblasts, and endothelial cells, they are unable to undergo adipogenic differentiation unlike MSCs (Herrera et al., 2006). The evidence suggests that LSCs are similar to MSCs but have undergone a partial commitment to hepatic cells, making them a multipotent progenitor population (Herrera et al., 2006).

1.3.1 Stem Cells as a Therapeutic Tool
Of course, the bigger picture to studying MSCs is their potential for improving clinical therapies. Initially, ideal therapeutic candidates were embryonic stem cells (ESCs) due to their pluripotency, however due to several technical and ethical issues, such as potential teratoma formation and the derivation of ESCs from human
embryos, it was necessary to pursue alternative avenues of research (Undale et al., 2009). In 2006, Takahashi and Yamanaka took adult fibroblasts and by retrovirally introducing the genes for 4 transcription factors (Oct3/4, Sox2, Klf4, c-Myc) caused these cells become similar to ESCs. These cells, called induced pluripotent stem (iPS) cells, provide huge potential for studying pluripotent stem cells whilst alleviating any ethical issues associated with ESCs. MSCs are adult stem cells and, other than the fact they are more easily obtained, another advantage is that they are immunomodulators. They actively inhibit T-cell proliferation, meaning immunosuppression of the host would not be necessary to allow an allogeneic MSC transplantation (Tse et al., 2003). The use of MSCs as a treatment has been studied extensively, for disorders including bone breaks, bone diseases, neurodegenerative diseases, musculoskeletal conditions, stroke, myocardial infarction, and cancer (Weiss et al., 2006; Cheng et al., 2008; Sonabend et al., 2008; Undale et al., 2009; Richardson et al., 2010; Eckert et al., 2013).

The use of marrow cells to heal a broken mouse femur has already been described (Ohgushi et al., 1989a) and Undale et al. (2009) describe how human MSCs have also benefitted patients with osteogenesis imperfecta, hypophosphatasia, and fracture non-union. Human MSCs have been of benefit when injected into the brains of Parkinson’s disease model rats, reducing the number of apomorphine-induced rotations (Weiss et al., 2006). With respect to cancers, malignant gliomas are highly infiltrative, therefore transgene delivery is problematic, with direct injections being fairly ineffective (Sonabend et al., 2008). However, studies using human MSCs infected with an oncolytic adenovirus show that they can migrate in vitro to glioma cells. When injected away from the tumour in vivo, the MSCs still migrate to the tumour and are 46-fold more effective at viral delivery and tumour infection (Sonabend et al., 2008). Investigations into myocardial infarctions show improvement to cardiac performance after an intravenous infusion of MSCs which overexpress the chemokine receptor 4 (CXCR4) (Cheng et al., 2008). MSCs overexpressing CXCR4 migrate toward the myocardium in greater numbers and with more efficiency than normal MSCs (Cheng et al., 2008). Compared to control rats, the CXCR4-MSC treated rats showed significant myocardial repair with a decrease
in wall thinning and a preserved left ventricle chamber with no depression (Cheng et al., 2008). These examples show the huge potential that MSCs provide with regard to varying therapies.

As of July 2013, MSCs were being used in over 250 clinical trials to treat inflammatory diseases (clinicaltrials.gov; Levy et al., 2013). Previous studies have manipulated MSCs to express homing ligands or chemokine receptors in order to target particular areas (Cheng et al., 2008; Sackstein et al., 2008), and now that is being combined with mRNA transfection of the MSCs in order to provide a vehicle for therapeutic factors (Levy et al., 2013). Together this combination allows MSCs to target a specific disease area, e.g. site of inflammation, and secrete therapeutic factors, e.g. anti-inflammatory cytokine interleukin-10 (IL-10) (Levy et al., 2013).

1.3.2 Mesenchymal Stem Cell Niche within the Bone and Marrow

The idea of a specialised microenvironment, or niche, was first suggested by Schofield (1978) and since then it has been identified as specific locations relative to each stem cell (Mohyeldin et al., 2010). These defined compartments provide both local and systemic factors to maintain quiescence of the stem cells, as well as providing a three dimensional highly specialised microenvironment made up of various cells, vasculature, and extracellular matrix (Scadden, 2006). It is critical for stem cell existence and provides the optimal environment for maintaining multipotency and self-renewal. The niche, which acts as a reservoir for the stem cells until they are required, provides cell-cell interactions, such as MSC and HSC contact, plus paracrine communication, for example via chemokines, which is important for stem cell survival (Scadden, 2006). In the bone marrow, endothelial cells and perivascular cells are vital for HSC maintenance. These niche cells express Scf (stem cell factor) and when knocked out from endothelial and perivascular cells specifically, HSCs are lost (Ding et al., 2012). This highlights the importance of the niche in stem cell maintenance.
Throughout the body there are varying types of stem cell and so, unsurprisingly, there are also varying stem cell niche designs. These include a single layer of cells between the subventricular zone and lateral ventricle where the subventricular astrocyte stem cells reside; the bulge of the hair follicle, found under the sebaceous gland which is home to the multipotent skin stem cells; the base of each intestinal crypt which is the niche for intestinal stem cells; and the complex microenvironment of the bone marrow which is where the HSCs are found, supported by osteoblasts which line the trabecular bone (Figure 1.3.) (Fuchs et al., 2004).

![Figure 1.3.](image)

*Figure 1.3. shows the haematopoietic stem cell niche.* The HSCs (shown in green) reside here where contact with the osteoblasts, which line the trabecular bone (TB), is vital for their maintenance. (Image altered from Fuchs et al. (2004)).

The HSC niche in the bone is the microenvironment where the HSCs are found. In this niche, Spindle-shaped N-cadherin$^+$CD45$^-$ osteoblastic cells, or SNO cells, increase in number mirroring an increase in HSC number (Zhang et al., 2003). This correlation is partially due to their attachment, via the junction molecule N-cadherin and associated β-catenin, which are located on the SNO cells and on long term HSCs, as well as to BMP signalling (Zhang et al., 2003). It is thought that these SNO cells, which line the bone surface, are important in regulating niche size and supporting HSCs (Zhang et al., 2003).
As well as osteoblasts, other mesenchymal cells, including MSCs and mesenchymal progenitor cells (MPCs), are also involved in regulating the haematopoietic microenvironment and HSC maintenance in the endosteal niche (Sacchetti et al., 2007). MPCs include clonogenic CD146+ skeletal progenitors which regulate Ang-1 expression and associate with blood vessels to play a vital role in developing spatially distinct microenvironments in the bone marrow (Sacchetti et al., 2007). Nestin+ MSCs are found close to HSCs in the haematopoietic stem cell niche, and are required for HSC maintenance and survival (Méndez-Ferrer et al., 2010). The endosteal location of these MSCs relative to the HSCs and blood vessels are shown in the schematic diagram by Ehninger and Trumpp (2011) (Figure 1.4.).

Figure 1.4. shows the location of the HSC niche in trabecular bone cavities. HSCs (shown in yellow) are located near the osteoblast-lined endosteum and near to the local vascular sinusoids. They are in contact with nestin+ MSCs (shown in red) which promote HSC maintenance (Adapted from Ehninger and Trumpp, 2011).

As well as in the HSC niche in the bone, MSCs are found in other perivascular niches close to blood vessels, in essentially all tissues, and bear a resemblance to pericytes (Crisan et al., 2008). The environment of the niche is determined by architectural components such as cells and blood vessels, which also determine oxygen levels in these niches, as reviewed by Mohyeldin et al. (2010). Mohyeldin et al. (2010) discussed the importance of low oxygen levels for self renewal and
multipotency of various stem cells, including haematopoietic, mesenchymal, and neural stem cells. Blood in human bone marrow has an oxygen level of ~7%, and using this value mathematical models have shown that oxygen levels in the bone marrow itself range from ~1% to ~5%, from the inner bone surface to the sinuses respectively (Ishikawa and Ito, 1988; Chow et al., 2001; Antoniou et al., 2004). It is therefore unsurprising that the low oxygen levels in the bone marrow have been found to extend the lifespan of MSCs and allow them to keep their stemness, i.e. proliferate without differentiating (Fehrer et al., 2007). Compared to the marrow with its raised O₂ level, the numbers of MSCs found were 4-fold higher on the surface of the trabecular bone, where the O₂ levels are lower (Fehrer et al., 2007). The MSCs had a significantly increased proliferative lifespan when cultured at 3% O₂ in vitro. Furthermore, their ability to differentiate into adipocytes was reduced and differentiation into osteoblasts was not observed at all (Fehrer et al., 2007). However, when cultured at 20% O₂ these cells lost some of their stemness and were capable of differentiating (Fehrer et al., 2007).

1.3.3 Mesenchymal progenitors

When the bone marrow is cultured it can be split as mentioned earlier into haematopoietic and non-haematopoietic compartments. The haematopoietic cellular compartment is made up mainly of macrophages. Seshi et al. (2000) described the breakdown of bone marrow as roughly 35% macrophages, 5% other haematopoietic cells, and 60% non-haematopoietic cells. Many studies describe the non-haematopoietic cells as a heterogeneous population of mesenchymal stromal cells (Liesveld et al., 1989; Clark and Keating, 1995; Prockop, 1997). However, Seshi et al. (2000) challenge this belief by providing evidence that they may actually be a single cell type: a multidifferentiated MPC. Having been purified away from the macrophages, these multidifferentiated MPCs coexpress genes and markers specific for various mesenchymal lineages. They report 100% of the purified non-haematopoietic cells expressed two adipocyte markers, two fibroblast markers, and one osteoblast marker (Nile Red, Oil Red O, fibronectin, prolyl-4-hydroxylase, and alkaline phosphatase (ALP) respectively). More than 85% of these cells were also
positive for α-actin, the smooth muscle marker. With these data, Seshi et al. (2000) suggest that these cells are a single, multidifferentiated cell type coexpressing multiple markers for at least 4 different mesenchymal lineages. This was backed up with the coexpression of lineage specific mRNAs; including adipsin, cadherin-11, collagen type 1, decorin, and fibronectin (Seshi et al., 2000).

Notch signalling has been shown to play an important role in the maintenance of mesenchymal progenitors in the bone marrow, bone homeostasis, and growth plate maintenance (Hilton et al., 2008). Trabecular bone mass increases in adolescent mice with altered Notch signalling, with the accumulation of bone replacing most of the marrow cavity (Hilton et al., 2008). This occurs because of the overproduction of trabecular osteoblasts and the elongation of hypertrophic cartilage. These mice then went on to suffer from severe osteopenia (low bone mineral density) in later life (Hilton et al., 2008). Normal Notch signalling controls chondrocyte proliferation and maintains a reservoir of mesenchymal progenitors. It does this by inhibiting osteoblast differentiation via Hes1, Hey1, and HeyL proteins, which in turn interferes with the function of Runx2 (Hilton et al., 2008). Using a Notch1::Cre lineage tracing method with a Rosa26-LacZ reporter, Notch signalling was shown to occur in the osteoblast lineage. LacZ was found to be positive in the bone matrix and on the surface of the trabecular bone (Hilton et al., 2008).

In order to characterise and identify these cells, either as a multidifferentiated MPC or other, surface cell antigen expression profiles are commonly used. Outlined here are various studies identifying surface cell markers used to isolate osteoblast and chondrocyte progenitors, which have also been used experimentally during this project.

1.3.3.1 Osteoblast progenitors - CD166 (ALCAM) and Sca-1

CD166 or Activated Leukocyte-Cell Adhesion Molecule (ALCAM) is a marker expressed by MSCs (Bruder et al., 1998). The expression pattern of CD166 suggests an involvement in osteoblast formation; expression is present in the MSCs and
osteoblast progenitors, but once differentiation into mature osteoblast occurs and alkaline phosphatase (ALP), the osteoblast marker, is expressed, CD166 expression is lost (Bruder et al., 1998). CD166 which has 5 extracellular Ig domains and is part of the Ig superfamily, is expressed by activated T-cells, B-cells, and monocytes (Bowen et al., 1995). Bowen et al. (1995) also showed that CD166 is a ligand of CD6. Adhesion of CD6-expressing cells to T cells is mediated by CD166 and suggests that CD166-CD6 interactions may be involved in the regulation of T cell maturation (Bowen et al., 1995). As well as this, adhesive interactions between activated leukocytes are shown to be mediated by CD166-CD166 binding (Bowen et al., 1995). In addition to MSCs, CD166 is expressed by neuronal cells (Tanaka et al., 1991), endothelial and haematopoietic cells (Ohneda et al., 2001), and haematopoiesis-supporting osteoblasts (Nelissen et al., 2000), with the human homolog of CD166 (HCA) being expressed on HSCs and myeloid progenitors in the bone marrow (Uchida et al., 1997). Arai et al. (2002) showed that CD166+ cells from the perichondrial region can differentiate to form osteoblasts, adipocytes, and chondrocytes, as well as support the formation of osteoclasts from bone marrow progenitors. The CD166-CD166 binding previously mentioned is also a mediator of endothelial and perichondrial cell adhesion promoting vascularisation of the cartilage (Arai et al., 2002). These findings suggest that CD166+ MSCs are present in the perichondrial region and provide bone and cartilage formation during endochondral ossification (Arai et al., 2002). The properties of CD166 make it a good marker for osteoblast progenitors in the developing limb.

Sca-1 (also called lymphocyte activation protein-6A (Ly-6A)) is a glycosyl phosphatidylinositol or GPI-linked cell surface protein (GPI-AP) from the Ly6 gene family and is needed for normal HSC activity, self-renewal, and development of committed progenitors (reviewed by Holmes and Stanford, 2007). When Sca-1 is knocked out mice show a decrease in megakaryocyte and platelet production, and are also defective in transplantation abilities; both short-term competitive repopulations and serial transplantations (Ito et al., 2003). Sca-1+/− HSCs also show defects in c-kit expression, colony formation, and homing ability to bone marrow (Bradfute et al., 2005). Sca-1 is found on the surface of various stem cells including haematopoietic
(Ito et al., 2003), mammary gland (Welm et al., 2002), cardiac (Oh et al., 2003), and as discussed previously mesenchymal (Baddoo et al., 2003; Morikawa et al., 2009). Consequently, it is often used to isolate various stem cells.

Nakamura et al. (2010) characterised a subpopulation of non-haematopoietic and non-endothelial (CD45−CD31−Ter119−) adult bone marrow cells from the endosteum by the expression of Sca-1 and CD166. They showed an enrichment of osteoblasts in the CD166−Sca-1− and CD166+Sca-1− populations, whereas the CD166+Sca-1+ cells were enriched for immature mesenchymal cells (Nakamura et al., 2010). The CD166+Sca-1+ cells express MSC-associated genes and are capable of differentiation into osteoblasts and adipocytes, however these cells have very low chondrogenic potential (Nakamura et al., 2010). Therefore the CD166+Sca-1− cells are a progenitor population with restricted differentiation capabilities. The two Sca-1 negative populations positively stain for ALP meaning both these populations contain osteoblasts, as well as having osteogenic potential (Nakamura et al., 2010). This is backed up by Runx2 expression. Osteopontin (Opn) and Osteocalcin (Ocn) are expressed but expression varies between the two populations; Opn is higher in CD166+Sca-1− cells and Ocn higher in CD166+Sca-1−, suggesting that the CD166+Sca-1− population contains more differentiated mature osteoblasts. With regard to adipogenesis, CD166+Sca-1− cells showed lipid accumulation, but CD166− Sca-1− did not. The chondrogenic properties were also evaluated and showed alcian blue staining and type X Collagen expression in the CD166+Sca-1− cells suggesting that this population also contains chondrocytes (Nakamura et al., 2010).

### 1.3.3.2 Chondrocyte progenitors – CD29 and CD146

The skeletal muscle fascia, or fibrous connective tissue, has been used to characterise chondrogenic populations using 2 cell surface markers: CD29 and CD146 (Li et al., 2011).

CD29 is a cell surface marker for mesenchymal stromal cells as covered earlier, and is found in various stromal tissues including the bone marrow stroma (Baddoo et al.,
CD29 is also found on chondrogenic progenitors; CD29+ cells in synovial tissues present with MSC characteristics and chondrogenic capacities (Gimeno et al., 2005). CD146 (or S-endo1, Mel-CAM, Muc18, or gicerin) is a surface glycoprotein and member of the immunoglobulin superfamily (Lehmann et al., 1989; Sers et al., 1993). It is a cell surface marker for endothelial cells, smooth muscle cells, pericytes, and other vasculature-related cells (Li et al., 2003; Middleton et al., 2005; Duda et al., 2006). Three populations from the fascia were studied: CD29+CD146−, CD29−CD146+, and CD29−CD146−, (CD29−CD146+ were not found) (Li et al., 2011). The first 2 populations showed chondrogenic differentiation potential, but not the double positive CD29+CD146+ (Li et al., 2011). This suggests that the CD146+ vasculature related cells possess the least chondrogenic potential. The same results were also seen in the endomysium and perimysium of the skeletal muscle (Li et al., 2011). These skeletal muscle derived cells are similar to the bone marrow stromal cells as they both have mesenchymal origins, the ability to form colonies, and have fibroblastic morphologies (Mastrogiacomo et al., 2005). They also express markers of the osteogenic lineage; ALP, OPN, and osteonectin (Mastrogiacomo et al., 2005). Mineralised bone matrix was formed by these cells in vitro and bone tissue formed in vivo (Mastrogiacomo et al., 2005). Skeletal muscle derived cells resemble bone marrow stromal cells and therefore the use of CD29 and CD146 should be appropriate for the isolation of a chondrogenic population.

1.4 Overview of Wt1 – a mesodermal / mesenchymal regulator

The Wilms’ tumour gene, WTI, found on human chromosome 11 (band p13), has 10 exons, and encodes a protein with four C-terminal zinc finger domains, characteristic of transcription factors (Call et al., 1990). Mutation of this tumour suppressor gene in humans can lead to the eponymous paediatric kidney tumour, kidney glomerulosclerosis, and male to female sex reversal due to gonadal dysgenesis, found in both Denys-Drash syndrome and Frasier syndrome, and occasionally heart problems (Hohenstein and Hastie, 2006; Chau and Hastie, 2012). So far alternative splicing, alternative start codons, and RNA editing can theoretically produce up to 36
isoforms, represented in Figure 1.5. (Hohenstein and Hastie, 2006). The KTS isoforms are the most well studied variations where three amino acids (KTS) are either present or absent between the third and fourth zinc fingers of the protein (Hohenstein and Hastie, 2006). These ±KTS isoforms are also the only variations known to be conserved in all vertebrates (Hohenstein and Hastie, 2006). Dallosso et al. (2004) identified the AWT1 transcript which results from an alternative exon 1, the promoter of which, unlike the WT1 promoter, is imprinted and only expresses the paternal allele.

As a transcription factor, only the −KTS isoforms which make up a third of all WT1 proteins, have high affinity binding to DNA and are therefore more active in the regulation of transcription (Hohenstein and Hastie, 2006). There is a large body of evidence suggesting that WT1 also functions in post-transcriptional processes, through binding to RNA and proteins involved in RNA splicing. The +KTS isoforms are particularly implicated in RNA binding and splicing (Larsson et al., 1995; Davies et al., 1998 – see reviews). Due to its roles in transcription, and also RNA processing, WT1 is mainly located in the nucleus (Niksic et al., 2004). However WT1 (±KTS) is also known to be a shuttling protein, moving back and forth between the nucleus and the cytoplasm meaning that a proportion of endogenous WT1 protein can be found in the cytoplasm (Niksic et al., 2004).
The biology of WT1 can often be contradictory. This is apparent when looking at its role in mesenchymal to epithelial transition (MET) and epithelial to mesenchymal transition (EMT). EMT and MET are important processes during gastrulation and embryo development (Acloque et al., 2009). Epithelial cells are immobile with an apical-basal polarity and form basement membranes. Mesenchymal cells on the other hand, have no polarity and unlike epithelial cells are mobile. Interestingly, these 2 cell types are able to switch from one to the other (reviewed by Chau and Hastie, 2012). Via EMT, cells become mobile and are able to move to alternative locations, which is also how cancer cells act when migrating around the body (Acloque et al., 2009). Both mesenchymal and epithelial cells express characteristic proteins. For example, E-cadherin is expressed by epithelial cells and is important for initiating adhesive contacts. As well as proteins, transcription factors are important for EMT/MET, with Snail transcription factors down regulating E-cadherin and consequently promoting EMT (reviewed by Chau and Hastie, 2012). EMT and MET oscillates throughout embryo and organ development (Pérez-Pomares and Muñoz-Chápuli, 2002). This is summarised in Figure 1.6.

Figure 1.6. shows the cyclical nature of EMT and MET (Chau and Hastie, 2012).
During development mesenchymal cells in the ureteric bud undergo MET to form nephrons (Davies et al., 2004). siRNA applied to a murine kidney organ culture shows that Wt1 expression is required for this MET (Davies et al., 2004). The siRNA represses Wt1 expression which results in a lack of nephron formation, at the pre-epithelial stage (Davies et al., 2004). The repression of Wnt4 using siRNAs also showed the same lack of nephrogenesis without altering Wt1 expression levels, suggesting that Wt1 is upstream of Wnt4 (Davies et al., 2004). Genetic proof that Wt1 is required for the kidney MET was reported by the Hastie laboratory who showed that deletion of a floxed Wt1 allele in kidney mesenchyme led to failure of the MET and nephron formation (Essafi et al., 2011). However, in heart development the opposite is seen as Wt1 is required for EMT (Martínez-Estrada et al., 2010). Mesenchymal progenitor cells are formed when the epicardial cells undergo EMT. An epicardial-specific Wt1 knockout resulted in a loss of these mesenchymal progenitor cells (Martínez-Estrada et al., 2010). Snail1 and Cdh1 genes, both required for EMT, are directly regulated by Wt1. These genes and their respective proteins, Snail and E-cadherin, both have altered expression in the Wt1 knockout; Snail expression decreases and E-cadherin increases (Martínez-Estrada et al., 2010). The paradoxical role of Wt1 in EMT and MET has recently been explained by the “chromatin flip-flop” mechanism (Essafi et al., 2011). This mechanism shows that Wt1 dichotomously regulates Wnt4; directly activating it in the kidney, but repressing it in the epicardium (Essafi et al., 2011). Wt1 does this by switching or “flipping” the chromatin state of the entire CCCTC-binding factor (Ctcf)-bound Wnt4 locus, but without affecting the surrounding regions (Essafi et al., 2011). This is only observed during development. It is, however, still unclear as to whether EMT/MET is important in normal adult homeostasis.

A second contradictory aspect of WT1 is the role it plays in cancer. The embryonic kidney tumour, Wilms’ tumour, is the most common renal tumour found in children, occurring around once in every 10,000 live births (Menke et al., 1998). WT1 functions as a tumour suppressor where WT1 mutations result in the development of Wilms’ tumours (Hohenstein and Hastie, 2006). A study of the WT1 gene in 32 Wilms’ tumours identified one nonsense point mutation which changed an arginine
to a stop codon, and one missense exonic zinc finger point mutation which changed an arginine into a cysteine (Little et al., 1992). This missense mutation and amino acid swap occurs at a region of the gene important for specific DNA binding (Little et al., 1992). Mutations in WT1 are seen in patients who suffer from Wilms’ tumour with aniridia, genitourinary dysplasia and mental retardation (known as WAGR syndrome) as well as in the tumour cells of Wilms’ tumour without aniridia (Francke et al., 1979; Kaneko et al., 1981). However, when it comes to adult tumours, it is possible that WT1 may be acting as an oncogene. WT1 is highly expressed in the tumours of tissues in which it is not normally found. These include human colon cancers (Koesters et al., 2004), primary breast tumours (Loeb et al., 2001), desmoid or soft tissue tumours (Nik et al., 2005) and primary astrocytic tumours (Oji et al., 2004). To add to the evidence that WT1 expression results in tumourigenesis, the growth of cancer cell lines is inhibited by the treatment of WT1 antisense oligomers (Oji et al., 2004). In a similar experiment cell cycle arrest and apoptosis were shown to be decreased in breast cancer cells, but with the addition of WT1 antisense oligomers proliferation decreased, and cell cycle arrest plus apoptosis were induced (Tuna et al., 2005). This study shows that WT1 inhibits apoptosis, and promotes the cell cycle to S-phase proliferation, resulting in tumour formation (Tuna et al., 2005).

The fact that WT1 can cause either differentiation into mesenchymal cells or epithelial cells may go some way to explaining how it can also act as both a tumour suppressor and possible oncogene, depending on the tissue. This is highlighted by the adult cancers, as previously discussed, which become metastatic through EMT. On the other hand, the childhood Wilms’ tumours arise from mesenchymal cells which would normally undergo MET but are blocked from doing so (Hohenstein and Hastie, 2006).

1.4.1 Wt1 in Development

Wt1 is an important gene for the development of several tissues and is shown to be a key regulator of the mesenchyme to epithelial transition during this development (Armstrong et al., 1993). Embryonic day 9 (e9) is the earliest expression of Wt1 in a
developing mouse embryo (Armstrong et al., 1993). At this stage it is expressed in the intermediate mesenchyme lateral to the coelomic cavity, and soon after in the urogenital ridge and differentiating heart mesothelium (Armstrong et al., 1993). At e9.5, *Wt1* expression is observed in the septum transversum mesenchyme which is the source of the epicardium, the diaphragm, hepatic sinusoids, and mesothelium of the liver (Chau and Hastie, 2012). Two days later *Wt1* expression is seen in the metanephric mesenchyme and spinal cord (e11). *Wt1* is then expressed in the induced kidney mesenchyme (e12.5), differentiating body wall musculature (e13.5), and then in the fourth ventricle of the brain (e15.5) (Armstrong et al., 1993). This expression pattern was also seen very similarly in human embryos between 28 and 70 days (equivalent of e10 to e15 in mouse) (Armstrong et al., 1993). Another study showed that 6-7 week (42 to 49 day) human embryos express *WT1* in the metanephric blastema, glomerular epithelium, and developing gonad (Pritchard-Jones et al., 1990). In the murine urogenital system, *Wt1* is expressed in the gonads from as early as e12.5 (Pelletier et al., 1991). Interestingly, expression levels vary depending on the sex of the mouse. In the females, *Wt1* expression is steady from day 4 after birth to day 40 in the reproductive system (Pelletier et al., 1991). However, once a male is born, *Wt1* expression steadily increases in the testis, especially the Sertoli cells, peaking at day 8 postpartum, followed by a slight decrease throughout maturation (Pelletier et al., 1991).

The *Wt1* gene is essential for early stages of kidney development, as well as being expressed in the gonads, spleen, and mesothelium. Kreidberg et al. (1993) showed that homozygote mutants resulted in failed kidney and gonad development due to apoptosis on day 11 of gestation. At day 12 of gestation the embryos became oedematous, before embryonic lethality occurred by resorption between days 13 to 15 of gestation. Homozygote mutants also show disrupted development in other tissues including the diaphragm, lungs, and especially the heart, which is the most likely cause for early embryo death (Kreidberg et al., 1993). In the heart, *Wt1* is required for generating mesenchymal progenitors for coronary vasculature, which arise from the epicardium (Martínez-Estrada et al., 2010).
Similar to the epicardium, which is a mesothelial coating of cells, the mesothelial cells of the intestine and lung also express *Wt1* (Wilm *et al.*, 2005; Que *et al.*, 2008). In the developing gut, the coelomic epithelium (which gives rise to the mesothelium) expresses *Wt1* at e10 (Carmona *et al.*, 2013). It is possible that this *Wt1* expression is responsible for triggering the EMT of these coelomic epithelium cells into mesenchymal mesothelium (Carmona *et al.*, 2013). A lineage tracing model showed that these *Wt1* positive methothelial cells differentiate and contribute to the vascular smooth muscle of the gut, mesenteries, Cajal and Cajal-like interstitial cells, and other intestinal tissues (Wilm *et al.*, 2005; Carmona *et al.*, 2013). From e10.5 and onwards in the developing lung, *Wt1* is expressed in the mesothelial cells which coat the trachea and lung surface (Que *et al.*, 2008). Using *in vivo* lineage tracing of the lung mesothelium these *Wt1* positive cells contribute to pulmonary blood vessel wall cells, positive for smooth muscle actin and PDGFR-β (Que *et al.*, 2008). Further lineage tracing studies show that these *Wt1* positive mesothelial cells, which line the pulmonary buds, give rise to parts of several other tissues in the developing lung (Cano *et al.*, 2013). These include the bronchial musculature, bronchial and tracheal cartilage, interstitial cells, as well as some of the pulmonary endothelial and smooth muscle cells (Cano *et al.*, 2013).

### 1.4.2 Wt1 in Adult Homeostasis

Compared with during development, *Wt1* is expressed in a small number of cells in only a few adult tissues. These tissues include the mesothelium, gonads, and kidney. A study of *Wt1* in adult rats identified expression in the testis, spleen, kidney, and mesothelium (Walker *et al.*, 1994). The Sertoli nurse cells in the testis express *Wt1*, and it plays a functional role in communication between the Sertoli and germ cells which results in spermatogenesis (Rao *et al.*, 2006). As well as the Sertoli cells of the testis, *Wt1* expression is also seen in the corresponding granulosa cells of the ovaries (Pelletier *et al.*, 1991). Expression of *Wt1* continues from development through to adulthood in the kidneys, specifically in the podocyte cells of the glomerulus, plus some low levels in the adult eye and tongue (Armstrong *et al.*, 1993). *Wt1* expression levels have also been measured in adult human bone marrow,
specifically the CD34+ haematopoietic progenitor cell population, of which 1.2% express \( Wt1 \) (Hosen et al., 2002).

Recent studies in the Hastie laboratory by Dr You-Ying Chau have shown that \( Wt1 \) is a major regulator of adult homeostasis. The ubiquitous knock out of \( Wt1 \) in the adult mouse results in multiple organ failure within the space of 9 days following the tamoxifen induced deletion (Chau et al., 2011). In addition to glomerulosclerosis and podocyte loss, the mice suffer from severe atrophy of the spleen and exocrine pancreas, failure to produce erythrocytes, decreased heart size, and rapid fat and bone loss. Chau et al. (2011) showed that \( Wt1 \) is expressed in oligolineage-restricted haematopoietic progenitor cells which are severely reduced in the bone marrow and spleen of the mutant mice. The volume of the trabecular bone decreased by 30% and the inner surface of the bone was ragged in the mutants compared to controls (Chau et al., 2011). Bone mass is a balance between synthesis from osteoblasts (arising from the mesenchymal stem/progenitors) and turnover by osteoclasts (arising from the haematopoietic progenitors). Chau et al. (2011) showed that there is an increase in osteoclasts within the mutant bone marrow, despite cells being less capable of osteoclast formation when deletion was carried out \textit{in vitro}. In addition, preliminary experiments showed that mutant mesenchymal cells had a reduced ability to make osteoblasts. Chau et al. (2011) also reported a decrease in the size of fat pads and fat vacuole size in the \( Wt1 \) mutant mice, as well as a decrease in the amount of adipocytes in bone marrow. As previously discussed, osteoblasts and adipocytes, along with chondrocytes, all differentiate from stromal MSCs found in the bone (Prockop, 1997). Interestingly, around 10% of Wilms’ tumours contain heterologous factors that include fat, bone, and cartilage as well as muscle (Royer-Pokora, 2003).

1.4.3 \( Wt1 \) and Oxygen Levels

\( Wt1 \) expression is observed in the myocardial blood vessels of ischemic rat hearts, caused by ligating the left coronary artery (Wagner et al., 2002). This led to studying \( Wt1 \) expression relative to oxygen levels by exposing rats to normobaric hypoxia (8% \( O_2 \)) for 8 hours which mimics the ischemia (Wagner et al., 2002). \( Wt1 \) mRNA levels were increased four-fold in the hypoxic hearts compared with controls.
(Wagner et al., 2003). Using the same technique to look at other organs showed both Wt1 protein and Wt1 mRNA levels are upregulated in hearts and kidneys of these rats compared with controls (Wagner et al., 2003). Normally Wt1 expression is only observed in the glomerular podocytes of kidneys, but interestingly under hypoxia it was also observed in the cytoplasm of the renal cortex tubular cells. The cytoplasmic location of the Wt1 protein suggests that, as well as acting transcriptionally, other mechanisms, such as nucleus-cytoplasm shuttling and RNA processing, are also being used. However, no change was seen in the brain or spleen. Further to this, hypoxia-inducible factor 1 (HIF-1) was found to regulate O2 dependent gene expression by binding to a hypoxia-responsive consensus element (HRE) of the Wt1 promoter (Wagner et al., 2003). This was identified after finding HIF-1α and Wt1 colocalisation in the ischemic myocardium (Wagner et al., 2002). HIF-1 is a heterodimeric transcription factor made up of HIF-1α and HIF-1β, both with basic helix-loop-helix DNA binding domains, and a common PAS domain (Wang et al., 1995). HIF-1α is regulated in an O2 dependent manner by proline hydroxylation of the oxygen-dependent degradation domain (ODD) (Ivan et al., 2001). Another phenotype of the hypoxic kidneys was the decrease in apoptosis shown by a reduction of TUNEL positive cells and the expression of BCL-2, the antiapoptotic protein (Wagner et al., 2003). Interestingly, Wt1 transcriptionally activates Bcl-2 (Mayo et al., 1999). Bcl-2 is expressed during development; at e12.5 there are high BCL-2 levels in the ureteric bud, metanephric cap, and tubular structure of the developing kidney (Novack and Korsmeyer, 1994). Mice deficient in Bcl-2 show enhanced apoptosis resulting in small kidneys with reduced nephron numbers (Novack and Korsmeyer, 1994).

As well as in vivo, Wt1 expression is also stimulated by hypoxia in vitro. Cells cultured at 1% O2 showed an increase in Wt1 expression, plus those cultured with CoCl2 and desferrioxamine (DFX) which mimic hypoxia by activating hypoxia-induced genes (Wagner et al., 2003). Interestingly, the cells cultured with CoCl2 and DFX also had increased expression of the HIF-1α protein (Wagner et al., 2003).
These observations show that \( Wt1 \) is sensitive to oxygen levels with HIF-1 activating the \( Wt1 \) promoter under hypoxic conditions, and this could be of importance with respect to Wt1 and its role in tumour growth due to intratumoural hypoxia. With regards to Wilms’ tumours, HIF-1α is coexpressed with vascular endothelial growth factor (VEGF) consistently across 18 human Wilms’ tumour specimens (Karth et al., 2000). As well as being colocalised with HIF-1α in Wilms’ tumours, VEGF expression is also very similar to \( Wt1 \) in the coronary vessels of the infarcted hearts (Wagner et al., 2002). VEGF is mainly involved in angiogenesis and neovascularisation, especially in hypoxic or ischemic tissues, and it is also a direct target of HIF-1α (as reviewed by Semenza, 1996). Together VEGF and HIF-1α are responsible for tumour vascularisation (Carmeliet et al., 1998).

As well as in tumourigenesis, Wt1 and hypoxia may also work together during organ development. HIF-1α is highly expressed in the developing kidney, specifically in the glomerular podocytes which are subject to microenvironmental hypoxia (Freeburg et al., 2003). As the developing kidney is also where we see high levels of \( Wt1 \) expression (Armstrong et al., 1993), it may be that the hypoxic condition of the developing kidney causes HIF-1 to stimulate \( Wt1 \). In concurrence with this, \( Wt1 \) is vital for heart development (Martínez-Estrada et al., 2010) as is hypoxia, i.e. lower oxygen levels in the fetus compared to the adult (Patterson and Zhang, 2010).

As mentioned previously, low oxygen levels are important for stem cells to maintain their self renewal properties and multipotency. Bone marrow \( O_2 \) levels vary between ~1% and ~5% (Ishikawa and Ito, 1988; Chow et al., 2001; Antoniou et al., 2004) and these low levels in the marrow extend MSC lifespan (Fehrer et al., 2007). This adds further interest to the involvement of Wt1 with hypoxia. Typically, most bone marrow studies (For example: Sun et al., 2003; Peister et al., 2004) are routinely carried out under standard culture incubator conditions of 5% \( CO_2 \) and 95% air (20% oxygen) which does not reflect the physiological conditions for most mammalian tissues, and as Fehrer et al. (2007) have shown, 3% \( O_2 \) is a more accurate condition for studying primary bone and marrow derived MSCs. Therefore, taking bone marrow oxygen levels plus oxygen-regulated \( Wt1 \) expression into account, the
culture studies in this investigation will be conducted at both normoxic (20% oxygen) and the more physiologically accurate hypoxic (3% oxygen) conditions.

1.4.4 Wt1 and IGF1

To investigate whether systemic factors regulated by Wt1 might be important for the phenotypes observed in the ubiquitous deletion, mutant and wild type serum were analysed using a chemokine array (Chau et al., 2011). This led to the observation, confirmed by ELISA, that circulating IGF-1 levels were reduced 85% in the mutant animals. Insulin-like growth factor 1 (IGF-1) is a peptide, encoded by the IGF-1 gene in humans, which is produced by several tissues and organs, mainly the liver, in response to growth hormone (LeRoith et al., 1992). It belongs to a family of proteins, binding proteins, and receptors which are essential for normal growth during developmental stages, from fetal to pubertal (Daughaday and Rotwein, 1989). Importantly, in the context of this study, IGF-1 signalling is required for osteoblast differentiation, shown by an osteoblast-specific knockout of the IGF-1 receptor gene (Zhang et al., 2002). This resulted in a decrease in the number of osteoblasts and a decrease in the rate of bone formation. IGF-1 is important in regulating differentiation of bone marrow derived MSCs into chondrocytes (Longobardi et al., 2006). The loss of bone phenotype were also seen by Yakar et al. (2002) in their double mutant mice for liver IGF-1 and binding protein acid labile subunit. The reduction in fat and bone seen by Chau et al. (2011) is probably due in part to systemic and local loss of IGF-1.

1.4.5 Wt1 and Chemokines

A cytokine array, consisting of 40 cytokines and 38 adipokines, was carried out and showed that a systemic inflammatory response was not the reason for the ubiquitous Wt1 deletion’s dramatic phenotypes (Chau et al., 2011). However, Wt1 does seem to be involved in regulating chemokines in the developing heart (Velecela et al., 2013). Knocking out Wt1 in an in vitro epicardial cell line resulted in a significant increase in chemokines Ccl5 and Cxcl10 (of the cysteine-X-cysteine (CXC) family) (Velecela et al., 2013). Chemokines and cytokines are involved in the chemoattraction of cells,
manipulating their migration for both inflammatory and homeostatic reasons (Raman et al., 2011). Both Ccl5 and Cxcl10 attract a variety of immune cells, including T cells, macrophages, natural killer (NK) cells, and dendritic cells (Raman et al., 2011), but functional assays have also highlighted their ability to regulate epicardial cell migration as well as cardiomyocyte proliferation (Velecela et al., 2013). Wt1 normally represses the transcription of Cxcl10 and Ccl5 directly, and indirectly by regulating the IRF7 gene (Velecela et al., 2013). IRF7, which transcriptionally regulates Cxcl10, is also upregulated in the Wt1 knock out, both transcriptionally and at a protein level (Velecela et al., 2013). The fact that this was not restricted to the heart but also observed in the kidney, suggests it could have a broader relevance.

Cxcl10 plays an important role in recruiting inflammatory cells to areas of damaged or infected tissue (Christen et al., 2003; Hintermann et al., 2010). Cxcl1 transcription is positively regulated by transcription factors and regulatory molecules including NF-κB, PARP-1, and CBP, and also negatively modulated by CAAT displacement protein (reviewed by Amiri and Richmond, 2003). Cxcl1 is an important chemokine in the inflammatory processes as it is a chemoattractant for neutrophils (Wang et al., 2002), however it may also play a role in bone biology. Cxcl1 is involved in both osteoblast and osteoclast formation which consequently determines bone physiology (Onan et al., 2009). In response, osteoblasts and osteoclasts also secrete chemokines, including Cxcl1, to regulate bone formation and resorption (Yu et al., 2004). Hence one of the aims of this project is to test for misregulation of such chemokines in the Wt1 mutant mesenchymal population. As mentioned previously, paracrine communication via chemokines is important in MSC/HSC interaction and survival (Scadden, 2006). Therefore it would also be interesting to look at Wt1 mutant chemokine expression and relate it to the MSC population.

1.4.6 Wt1 in tissue damage and repair

Aside from the eponymous childhood tumour and various adult cancers, Wt1 expression is seen in other diseases. It seems that Wt1 is activated in various
damaged tissues and may be playing a role in tissue repair; this is highlighted by studies of the liver and the heart (Chau and Hastie, 2012). During development (e13.5) Wt1-null mice are missing one of the 3 main lobes from their livers (Ijpenberg et al., 2007). While Wt1 is not expressed in hepatocytes, it is expressed in stellate cells derived from the mesothelium which is reduced in mutant mice (Ijpenberg et al., 2007). Hepatic stellate cells are a mesenchymal cell type and have been described as the liver’s equivalent to pericytes (Sato et al., 2003; Bergers and Song, 2005). This suggests that these stellate cells are secreting factors needed for hepatocyte growth. In the case of the developing heart, deleting Wt1 in the epicardium results in thinner ventricles and reduced cardiomyocytes, probably due to a paracrine epicardial effect (Martínez-Estrada et al., 2010). This highlights a certain parallel to the liver stellate cells and suggests that these epicardium derived cells are needed for cardiomyocyte growth. Another similarity is that both stellate cells and epicardial derived cells seem to share a similar molecular pathway, producing retinoic acid, which is transcriptionally activated by Wt1 (Chau and Hastie, 2012).

As covered earlier Wt1 expression is activated in the heart during ischemia (Wagner et al., 2002). Wt1 positive epicardial cells have also been found to proliferate and differentiate into functional cardiomyocytes possibly explaining how the damaged heart tissue initiates a damage repair response (Smart et al., 2011). It is well known that the liver is capable of fully regenerating lost tissue as well as damaged tissue (Michalopoulos and DeFrances, 1997). During hepatocyte regeneration and fibrotic response, stellate cells are activated and involved in this fibrosis-associated repair (as reviewed by Michalopoulos and DeFrances, 1997). A lineage tracing study has recently shown that Wt1 expressing mesothelial cells migrate from the surface of the liver, inwards, and generate hepatic stellate cells (Li et al., 2013). The lineage tracing model also showed that, depending on the damage of the liver, mesothelial cells can give rise to myofibroblasts as well as hepatic stellate cells (Li et al., 2013). Stellate cells also contribute to regenerating the lost liver by secreting various hepatic mitogens (Friedman, 2008).
1.4.7 Wt1 and MSCs

There are compelling parallels between different Wt1 expressing mesenchymal cell populations that suggest links with mesenchymal stem/progenitor cells. As previously discussed, mesothelial cells in the developing liver which express Wt1 are stellate cell precursors (Ijpenberg et al., 2007; Asahina et al., 2011; Li et al., 2013). Chau et al. (2011) showed Wt1 was also expressed in the pancreatic mesothelium and in the pancreatic stellate cells. The parallels between pancreatic and hepatic stellate cells suggest that pancreatic stellate cells may also be arising from the mesothelium, via EMT (Masamune and Shimosegawa, 2009; Chau et al., 2011; Li et al., 2013). In the liver and pancreas, stellate cells play an important role in cytokine synthesis and regulation of tissue fibrosis (Friedman, 2008; Masamune and Shimosegawa, 2009). Stellate cells are also positive for nestin (Lardon et al., 2002), linking them with the nestin+ MSCs (Méndez-Ferrer et al., 2010). Hepatic stellate cells have spindle-shaped bodies (Friedman, 2008) which are similar in appearance to the bone marrow cells cultured under MSC favouring conditions. Stellate cells may originate from both the mesothelium and bone marrow derived HSCs and could possibly be another subset of the Wt1 expressing cells (Miyata et al., 2008; Asahina et al., 2011). Hepatic stellate cells share striking similarities with pericytes (Sato et al., 2003; Bergers and Song, 2005). A FACS sorted pericyte population from the bone marrow was shown to include cells with mesenchymal stem cell properties (Crisan et al., 2008). In the kidney, podocytes, which similarly to pericytes and stellate cells, also have a spindle shaped appearance due to their long processes or ‘feet’, express both Wt1, which is a podocyte regulator (Guo et al., 2002) and nestin (Bertelli et al., 2007). Nestin expression in the developing kidney and heart overlaps with that of Wt1 (Wagner et al., 2006). A loss of Wt1 expression in these tissues results in drastically reduced nestin expression and suggests that in these organs nestin may be regulated by Wt1 (Wagner et al., 2006).

A recent study looking at the relationship between MSCs and the mesenchymal properties of Wilms’ tumours with Wt1 mutations, showed that a Wilms’ tumour cell line expresses surface proteins specific to human MSCs including CD105, CD90,
and CD73 and are able to differentiate into chondrocytes, osteoblasts, and adipocytes (Royer-Pokora et al., 2010). These findings also support the involvement of Wil in mesenchymal stem cell biology.

However, the most striking phenotype to support Wil’s involvement with mesenchymal lineages is the effect of Wil deletion shown by Chau et al (2011). Mutants showing drastic bone and fat loss, both of which are mesenchymal derivatives, strongly suggests Wil is playing a functional role in the homeostasis and maintenance of these mesenchymal tissues, and may even act as a functional marker for mesenchymal stem or progenitor populations.

1.5 Summary

There is considerable topical interest in tissue stem and progenitor cells, particularly with regard to their potential use in therapy. Bone marrow is largely split into two halves; the haematopoietic and the non-haematopoietic. The haematopoietic component consists of HSCs, haematopoietic progenitor cells, and their derivatives, the majority of which are macrophages. The non-haematopoietic component is made up mainly of endothelial and mesenchymal stem cells (MSC) plus their derivatives, including adipocytes, osteoblasts and chondrocytes (Tocci and Forte, 2003). It has been much more difficult to define mesenchymal stem and progenitor cells for different lineages than it has been for the haematopoietic compartment.

A recent deletion of tumour suppressor gene Wil in adult mice resulted in severe phenotypes amongst which were drastic loss of fat and bone, both of which are MSC derivatives (Chau et al., 2011). This shows that Wil expression is functionally important for the genesis of the mesenchymal lineages and also begs the question as to whether Wil is expressed in a subset of these mesenchymal stem or progenitor cells.
1.5.1 Hypothesis

The main goal of my thesis was to test the hypothesis that \( Wt1 \) is expressed in, and required for the function of, mesenchymal stem or progenitor cell populations within the bone marrow.

1.5.2 Aims

The aims of this project were to determine the role of \( Wt1 \) in the bone marrow with regard to mesenchymal stem/progenitor cell lineages; to characterise the \( Wt1 \) expressing population of cells and to determine the role of \( Wt1 \) in the maintenance and / or differentiation of these cells.

I also hoped to explore the molecular mechanisms by which \( Wt1 \) may regulate these mesenchymal populations and their interactions with the haematopoietic compartment.

In short, the aims of this PhD project have been:

- To isolate and characterise the BM-MSC population
- To characterise the Wt1 positive sub-population of BM-MSCs
- To examine the effect of hypoxia on these cells
- To examine the effect of \( Wt1 \) deletion on these cells
- To start investigating their interaction with surrounding cells
Chapter 2

Materials & Methods
2.1 General Reagents

The following solutions were prepared by the Core Scientific Services of the MRC IGMM Human Genetics Unit (HGU):

**PBS**
Phosphate Buffered Saline (pH 7.3): 8 g Sodium Chloride NaCl, 0.2 g Potassium Chloride KCl, 1.15 g Disodium Hydrogen Phosphate Na$_2$HPO$_4$, 0.2 g Potassium Dihydrogen Phosphate KH$_2$PO$_4$, made up in 1 litre distilled water. This is made up by dissolving 10 Dulbecco’s tablets in 1 litre of distilled water (Cat #BR0014, Oxoid Limited, Hampshire, RG24 8PW, UK).

**0.5 M EDTA**
186.1 g Ethyldiaminetetra-acetic acid di-sodium salt was dissolved in 1 litre sterile water, with solid NaOH (~20 g) added to pH 8.

**TBE Buffer (20x stock solution)**
216 g Tris Base, 110 g Boric Acid, 80 ml 0.5 M EDTA (pH 8), made up to a final volume of 1 litre with distilled water. For 1x TBE Buffer: 50 ml of 20x TBE in a final volume of 1 litre with distilled water.

**Trypsin**
2 g Trypsin (1:250), 5 ml Phenol Red, 0.06 g Penicillin, 0.13 g Streptomycin, made up to 1 litre in PBS and altered to pH 7.8 using NaHCO$_3$.

**Versene**
0.4 g Sodium EDTA, and 5 ml 0.2% Phenol Red made up to 1 litre with PBS.

**Penicillin/Streptomycin (P/S)**
7 g (10x10$^8$ units) Penicillin and 13 g Streptomycin, made up to 1 litre in distilled water.

**L-Glutamine**
30 g L-Glutamine dissolved in 1 litre of distilled water.
2.2 Transgenic Animal Models

2.2.1 Animal Husbandry

Mice were housed in two facilities: The Transgenic Unit (TGU) which is a barrier facility for animals created by transgenic approaches, and the Biomedical Research Facility (BRF) which is a semi-barrier facility for all other mice. All procedures were carried out under a Personal and Project Home Office licence where needed.

2.2.2 Genotyping of Mice

Ear clips were taken in order to identify the mice and to provide genomic DNA for PCR genotyping. Ear clipping and genotyping were carried out by Anna Thornburn from the MRC Human Genetics Unit (HGU).

2.2.3 WER mouse line

A Wt1 conditional line was generated in our group where the first exon of Wt1 is flanked by loxP sites to give Wt1<sup>loxP/loxP</sup> mice (Martínez-Estrada et al., 2010). These were crossed with Cre-ER<sup>TM</sup> mice where Cre is driven by the CAGG promoter, to give CAGG-CreER<sup>TM</sup>;Wt1<sup>loxP/loxP</sup> mice with a tamoxifen inducible Wt1 deletion (Chau et al., 2011).

2.2.4 Wt1-GFP mouse line

A knock-in reporter green fluorescent protein (GFP) mouse (Wt1<sup>GFP/+</sup>) generated by Hosen et al. (2007) was used for this study. GFP is expressed under the endogenous transcriptional regulatory elements of the Wt1 gene (Hosen et al., 2007).

2.2.5 WGER mouse line

The WGER mouse line was created by crossing a Wt1-GFP mouse with a WER mouse to give CAGG-CreER<sup>TM</sup>;Wt1<sup>loxP/GFP</sup> mice.
2.2.6 \textit{Wt1}^{CreERT2};\textit{mTmG} mouse line

To trace the lineage of \textit{Wt1} expressing cells a \textit{Wt1}^{CreERT2};\textit{mTmG} mouse strain was used (Chau \textit{et al.}, 2014). A knock-in mouse with a tamoxifen-inducible Cre recombinase at the \textit{Wt1} promoter (\textit{Wt1}^{CreERT2}) (Zhou \textit{et al.}, 2008) was crossed with an \textit{mTmG} double-fluorescent Cre reporter mouse (Muzumdar \textit{et al.}, 2007). In the \textit{Wt1}^{CreERT2};\textit{mTmG} mouse, Tomato is ubiquitously expressed under a pCA promoter, but following Cre mediated \textit{loxP} recombination and the removal of Tomato, GFP is expressed. Therefore, this allows cells expressing CreER (driven by the \textit{Wt1} promoter) to be “tattooed” at the time of tamoxifen induction. These GFP-expressing “tattooed” cells can now be traced as well as their progeny.

2.2.6.1 \textit{In Vivo} Lineage Tracing

To determine whether \textit{Wt1} positive cells are contributing to particular lineages Tamoxifen (Cat# T5648, Sigma-Aldrich Company Ltd., Dorset, England) was dissolved in Glyceryl Trioctanoate (Cat# T9126, Sigma-Aldrich Company Ltd., Dorset, England) at a concentration of 20 mg/ml. Either Tamoxifen or Glyceryl Trioctanoate was gavaged into the mice at a dose of 8 mg/40 g body weight. Adult mice given 4 doses (2 per week for 2 weeks) were harvested after 1 day, 3 days, 2 weeks, and 6 weeks from the final dose. Adult mice given 2 doses (2 per week) were harvested after 2 days. Maternal mice were gavaged with a single dose of 8 mg/40 g body weight at e14.5 and the offspring were analysed at e18.5, or fostered by CD1 mice until analysis at 10 days and 3 weeks.

2.2.7 Cre Control mouse line

To assess the toxicity of dissociated Cre we crossed pure C57BL/6J mice (The Jackson Laboratory, Maine, 04609, USA) with CAGG-CreER$^{TM}$ to give Cre$^+$ and Cre$^-$ mice.
2.2.8  *In Vivo* Deletion

Tamoxifen (Cat# T5648, Sigma-Aldrich Company Ltd., Dorset, England) was dissolved in Glyceryl Trioctanoate at a concentration of 20 mg/ml. Either Tamoxifen or Glyceryl Trioctanoate was intraperitoneally injected into the WGER mice at a dose of 4 mg/40 g body weight. Adult mice were injected for 5 consecutive days and harvested 10 days after the first injection.

2.2.9  *In Utero* Deletion

Tamoxifen (Cat# T5648, Sigma-Aldrich Company Ltd., Dorset, England) was dissolved in Glyceryl Trioctanoate at a concentration of 20 mg/ml. Either Tamoxifen or Glyceryl Trioctanoate was maternally gavaged into WGER mice at a dose of 8 mg/40 g body weight. Pregnant mice were gavaged at e12.5 with a single dose and pups were analysed at e18.5.

2.3  Tissue Culture

2.3.1  Isolation and Expansion of Murine MSCs

The Expansion Protocol for Cells Isolated from Bone Marrow was followed and found in the Technical Manual version 1.2.0 for Enumeration and Expansion of Mouse Mesenchymal Progenitor Cells Using MesenCult® (STEMCELL Technologies SARL, 38000 Grenoble, France).

The femur and tibia bones were removed from both hind legs of the mice. Using a 0.5 mm x 25 mm BD Microlance 3 needle (#300400, MidMeds Ltd., EN9 2HB), the bone marrow was flushed out of each bone into 8 ml Complete Media (45 ml minimum essential medium eagle, alpha modification (α-MEM) (Sigma-Aldrich Company Ltd., Dorset, England), 5 ml Fetal Bovine Serum (#SV30160.03, HyClone UK Ltd., Cramlington, NE23 1WA), 1 ml Pen/Strep (Penicillin and Streptomycin), and 0.5 ml Glutamine). A single cell suspension was created by adding 8 ml MesenCult® media and using a 0.8 mm x 40 mm BD Microlance 3 needle (#304432,
MesenCult® media was prepared using MSC Basal Medium (mouse) (#05501, STEMCELL Technologies SARL, 38000 Grenoble, France) and Mesenchymal Stem Cell Stimulatory Supplements (mouse) (#05502, STEMCELL Technologies SARL, 38000 Grenoble, France). For culture, the bone marrow cells in the media were divided into 2 sterile 6-well cell culture plates (#3516 Costar, Corning Incorporated, Corning, NY, 14841, USA), 2 ml/well for 4 wells. One plate was cultured under normoxic conditions (20% O₂) and the other under hypoxic conditions (3% O₂) in a Galaxy® 170R incubator (New Brunswick Scientific, Enfield, CT, USA).

After flushing the bone marrow cells from the femurs and tibias, the bones were crushed using a pestle and mortar. To increase the numbers of MSCs obtained from the crushed bones they were incubated at 37°C with Type 1 Collagenase (3 mg/ml) (#17100-017, Gibco®, Life Technologies Ltd, Paisley, PA4 9RF) in Dulbecco’s Modified Eagle Medium 1x (DMEM) (#41965-039, Life Technologies Ltd, Paisley, PA4 9RF) + 10% FBS for 1.5 hours (Morikawa et al., 2009). The cells were then strained through a cell strainer with a 40 μm nylon filter (#352340, BD Falcon™, Franklin Lakes, NJ, 07417, USA) and spun down at 277 Relative Centrifugal Force (RCF) using an Allegra X-22R Centrifuge (Beckman Coulter (UK) Ltd., High Wycombe, HP11 1JU) for 5 minutes. The cells were then resuspended in DMEM + 10% FCS + 2% P/S, before being plated out as per the marrow cells above.

After 2 days of culture, the cells were washed with PBS and fresh MesenCult® media provided. The mesenchymal cells should have proliferated and adhered to the plastic plates. Therefore, any remaining bone marrow stromal or haematopoietic cells will have been washed away. Media was changed for fresh MesenCult® media every 2-3 days, for 7 days. Following which, cells were trypsinized for 5 minutes (10ml trypsin in 50 ml versene) and pelleted.

If looking at the effects of Wt1 deletion, after 4 days of culture, 1 μM 4-OH tamoxifen ((z)-4-hydrotamoxifen, Cat# H7904, Sigma-Aldrich Company Ltd.,
Dorset, England) was added for 3 days to cause Cre mediated loxP recombination. Again the cells were trypsinized and pelleted.

2.3.2 Adipogenic Differentiation

Differentiation medium was made up in 45 ml of DMEM (#41965-039, Life Technologies Ltd, Paisley, PA4 9RF) and 5 ml FBS, with 0.1 ml 3-isobutyl 1-methylxanthine (IBMX 0.5 mM), (Cat# I5879, Sigma-Aldrich Company Ltd., Dorset, England) 0.05 ml Dexamethasone (DXM 1 µM) (Cat# D4902, Sigma-Aldrich Company Ltd., Dorset, England), and 0.05 ml Insulin (10 µg/ml) (Cat# I0516, Sigma-Aldrich Company Ltd., Dorset, England).

2.3.3 Osteogenic Differentiation

Osteogenic differentiation was carried out using the Mouse Mesenchymal Stem Cell Function Identification Kit according to the Handbook (Cat# SC010, R&D Systems Europe, Ltd., Abingdon, OX14 3NB, UK). Osteogenic supplement and α-MEM Basal Medium with P/S were added to cells for 14 – 21 days.

2.3.4 Chondrogenic Differentiation

Chondrogenic differentiation was carried out using the Mouse Mesenchymal Stem Cell Function Identification Kit according to the Handbook (Cat# SC010, R&D Systems Europe, Ltd., Abingdon, OX14 3NB, UK). Chondrogenic supplement and D-MEM/F-12 Basal Medium with P/S were added to cells for 17 – 21 days.

2.3.5 Colony forming unit fibroblast assay

CFU-F assays were carried out using bone and bone marrow prepared as previously described (See 2.3.1). Cell density of the pelleted cells was calculated using a haemocytometer and cells were plated out in 6 well culture plates at a density of 0.5 x 10^6 cells/well (2 ml of MesenCult® media per well). On the 3rd day of culture, once the cells adhered, cells were washed with PBS and then cultured with
MesenCult® plus 1 μM 4-OH Tamoxifen for 72 hours. Following this, cells were cultured with MesenCult® (minus tamoxifen) for 10 days, then washed with PBS. Colonies were incubated with 0.5% Cresyl Violet Acetate solution (Cat# C5042, Sigma-Aldrich Company Ltd., Dorset, England) (made in methanol and then filtered using Whatman® 113V Filter Paper) for 30 minutes at room temperature. After staining, colonies were washed in PBS three times and left to dry. Total colonies and large colonies were counted (colonies of 50 cells or more are categorised as large). Area and diameter of the colonies were measured using ImageJ software (http://rsbweb.nih.gov/ij/). See Appendix 1 for a schematic diagram.

The CFU-F assay was carried out using WGER mice and CAGG-CreER™;Wt1+/+ control strain mice.

2.4 Immunohistochemistry

2.4.1 Sample Preparation

Samples were fixed in 4% Paraformaldehyde in PBS (Cat# P6148, Sigma-Aldrich Company Ltd., Dorset, England) and then incubated in 14% EDTA disodium salt dehydrate (VWR International, Leicestershire, LE17 4XN) (pH 7.1) in PBS for 7 days to promote decalcification, making the bones easier to cut. Samples were then processed and embedded in paraffin wax using a Tissue-Tek VIP® Jr. Vacuum Infiltration Processor (Sakura Finetek UK Ltd., Thatcham, RG19 4LW, UK). Processing conditions and timings are outlined in Table 2.1.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Solution</th>
<th>Time for: Adult Leg (minutes)</th>
<th>e11.5 embryo</th>
<th>e13.5 embryo</th>
<th>14.5./ 15.5 embryo</th>
<th>e16.5 embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/V</td>
<td>Alternating pressure &amp; vacuum during processing</td>
<td>On after stage 5</td>
<td>Off</td>
<td>On after stage 5</td>
<td>On after stage 5</td>
<td>On after stage 5</td>
</tr>
<tr>
<td>1</td>
<td>70% EtOH</td>
<td>60</td>
<td>15</td>
<td>45</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>70% EtOH</td>
<td>60</td>
<td>15</td>
<td>45</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>85% EtOH</td>
<td>60</td>
<td>15</td>
<td>45</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>95% EtOH</td>
<td>60</td>
<td>15</td>
<td>45</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>100% EtOH</td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>100% EtOH</td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>Xylene</td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>Xylene</td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>Xylene</td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>Xylene</td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>11 - 14</td>
<td>Wax</td>
<td>60 per step</td>
<td>15 per step</td>
<td>45 per step</td>
<td>60 per step</td>
<td>90 per step</td>
</tr>
</tbody>
</table>

Paraffin sections were cut ~4-5 μm thick using a Leica RM2235 Rotary Microtome (Leica Biosystems Ltd., Newcastle Upon Tyne, NE12 8EW, UK), placed on SuperFrost® Plus Microscope Slides (#631-0108, VWR International, Leicestershire, LE17 4XN) and dried over night at 50°C.

2.4.2 H&E staining

Sections were dewaxed in Xylene (3x 5 minute washes). They were then rehydrated by a series of Ethanol washes (3x 2 minutes in 100% EtOH and followed by 1 minute in 90%, 70%, 50%, and 30% EtOH). Sections were washed in distilled water before 5 minutes stain with Mayer’s Hematoxylin (S3309, Dako UK Ltd., Cambridgeshire, CB7 4EX). Sections were washed in distilled water before differentiating with 1% HCl in 70% EtOH. Sections were then placed in Saturated Lithium Carbonate solution (5 seconds) and Eosin stain (2-5 minutes) with distilled water washes between. Eosin stain: 3 parts 1% aqueous Eosin and 1 part 1% EtOH,
with Acetic Acid 0.05% final concentration. Sections were dehydrated in 100% EtOH before clearing in Xylene (3x 5 minute washes). Slides were mounted using DPX mounting medium (SEA-0302-00A, CellPath Ltd., Powys, SY16 4LE) in a fume hood.

2.4.3 Solutions for paraffin immunoperoxidase and immunofluorescence

TEG Buffer pH9
1.211 g TRIS – (hydroxymethyl) aminomethane (#103156X, VWR International, Leicestershire, LE17 4XN), 0.19g EGTA (Ethylene glycol-bis (2-aminoethylether)-N,N,N’,N’- tetraacetic acid, Cat# E3889, Sigma-Aldrich Company Ltd., Dorset, England), made up to 1 litre in distilled water.

Wash 1 (0.01 M PBS, 1% BSA, 0.2% Gelatine, 0.05% Saponin, pH 7.4)
0.2 g Gelatin (Cat# G1890, Sigma-Aldrich Company Ltd., Dorset, England) and 20 ml 0.01 M PBS (pH 7.4) microwave heated until dissolved. Add this to 1 g Bovine Serum Albumin (BSA) (Cat# A3294, Sigma-Aldrich Company Ltd., Dorset, England), 0.05 g Saponin (Cat# S4521, Sigma-Aldrich Company Ltd., Dorset, England), dissolved in 80 ml 0.01 M PBS pH 7.4, to make 1 litre.

Wash 2 (0.01 M PBS, 0.1% BSA, 0.2% Gelatine, 0.05% Saponin, pH 7.4)
0.2 g Gelatin (Cat# G1890, Sigma-Aldrich Company Ltd., Dorset, England) and 20 ml 0.01 M PBS pH 7.4 microwave heated until dissolved. Add this to 0.1 g BSA (Cat# A3294, Sigma-Aldrich Company Ltd., Dorset, England), 0.05 g Saponin (Cat# S4521, Sigma-Aldrich Company Ltd., Dorset, England), dissolved in 80 ml 0.01 M PBS pH 7.4, to make 1 litre.

2.4.4 Immunoperoxidase – Paraffin sections

Sections were placed in Xylene overnight (or for a minimum of 2 hours). They were then rehydrated by a series of Ethanol washes (3x 10 minutes in 99% EtOH and 2x 10 minutes in 96% EtOH). Slides were blocked for 30 minutes for endogenous peroxidase (1 ml 30% H2O2 in 100 ml methanol), followed by a 10 minute 70%
EtOH wash and 3 washes in distilled water. Antigen target retrieval was carried out by placing 1 litre of TEG Buffer and the slides in a pressure cooker and boiling for 5 minutes. Once cooled, the slides were washed for 30 minutes in 50 mM NH₄Cl (made up in 0.01 M PBS, pH 7.4) and then 3x 10 minutes in Wash 1. Sections were incubated with primary antibody overnight at 4°C. Primary antibody concentrations are described in Table 2.2.

Following 3x 10 minute washes with Wash 2, the sections were incubated for 1 hour in the dark at room temperature with peroxidase conjugated antibody (described in Table 2.2), followed by a further 3x 10 minute washes with Wash 2. Slides were incubated in a fume hood for 10 minutes at room temperature with 3,3-diaminobenzidine tetrahydrochloride (DAB): 1 pH 7.0 DAB tablet (Cat# 4170, Kem-En-Tec Diagnostics, Taastrup, Denmark) dissolved in 10 ml distilled water, plus 10 μl 30% H₂O₂. After washing with PBS and distilled water, the sections were counterstained with Mayer’s Hematoxylin (S3309, Dako UK Ltd., Cambridgeshire, CB7 4EX) for 2 minutes, Acid Alcohol (1% HCl, 70% EtOH), Saturated Lithium Carbonate in H₂O, with washes of distilled water between. Slides were dehydrated by a series of Ethanol washes (2x 5 minutes at 70%, 96%, 99% EtOH, and Isopropanol) before clearing in xylene (3x 10 minutes). Slides were mounted using DPX mounting medium (SEA-0302-00A, CellPath Ltd., Powys, SY16 4LE) in a fume hood.

2.4.5 Dual Immunofluorescence – Paraffin sections

Sections were placed in Xylene overnight (or for a minimum of 2 hours). They were then rehydrated by a series of Ethanol washes (3x 10 minutes in 99% EtOH, 2x 10 minutes in 96% EtOH, and 1x 10 minutes in 70% EtOH), before a final wash in distilled water. Antigen target retrieval was carried out by placing 1 litre of TEG Buffer and the slides in a pressure cooker and boiling for 5 minutes. Once cooled, the slides were washed for 30 minutes in 50 mM NH₄Cl (made up in 0.01 M PBS, pH 7.4) and then 3x 10 minutes in Wash 1. Sections were incubated with primary
antibody overnight at 4°C. Primary antibody concentrations are described in Table 2.2.

Following 3x 10 minute washes with Wash 2, the sections were incubated for 1 hour in the dark at room temperature with the secondary antibodies (described in Table 2.2). After washing with PBS and distilled water, the sections were mounted using VectaShield Mounting Medium with DAPI (H-1200, Vector Laboratories, Inc., Peterborough, PE2 6XS, United Kingdom) and stored in the dark.

Table 2.2. Antibody Information

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Concentration</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GFP</td>
<td>Goat Polyclonal</td>
<td>1:1000</td>
<td>ab6673, abcam®, Cambridge, CB4 OFL, UK</td>
</tr>
<tr>
<td>WT1</td>
<td>Rabbit Polyclonal</td>
<td>1:1000</td>
<td>GTX15249, GeneTex, Inc., Irvine, CA, 92606, USA</td>
</tr>
<tr>
<td>Alexa Fluor® 488</td>
<td>Donkey Anti-Goat IgG</td>
<td>1:1000</td>
<td>A11055, Invitrogen Ltd, Paisley, PA4 9RF, UK</td>
</tr>
<tr>
<td>Alexa Fluor® 594</td>
<td>Donkey Anti-Rabbit IgG</td>
<td>1:1000</td>
<td>A21207, Invitrogen Ltd, Paisley, PA4 9RF, UK</td>
</tr>
<tr>
<td>Peroxidase-AffiniPure</td>
<td>Donkey Anti-Goat IgG</td>
<td>1:200</td>
<td>#705-035-003, Jackson ImmunoResearch Laboratories, Inc., West Grove, 19390, USA</td>
</tr>
<tr>
<td>Peroxidase-AffiniPure</td>
<td>Goat Anti-Rabbit IgG</td>
<td>1:200</td>
<td>#111-035-003, Jackson ImmunoResearch Laboratories, Inc., West Grove, 19390, USA</td>
</tr>
</tbody>
</table>

2.4.6 Imaging

Paul Perry and Matthew Pearson from the MRC HGU provided guidance and assistance with the imaging work conducted for this thesis. Immunoperoxidase and immunofluorescence stained sections were viewed using a Zeiss Axioplan 2 microscope (Carl Zeiss Ltd., Cambridge, CB1 3JS, UK). The Immunofluorescence imaging system comprises a Photometrics CoolSnap HQ2 CCD camera (Photometrics Ltd, Tucson, AZ), Zeiss Axioplan II fluorescence microscope with Plan-neofluar or Plan-Apochromat objectives, a 100W Hg source (Carl Zeiss, Welwyn Garden City, UK) and Chroma #89014ET three colour filter set (Chroma Technology Corp., Rockingham, VT). The single excitation and emission filters are
installed in motorised filter wheels (Prior Scientific Instruments, Cambridge, UK). Image capture was performed using in-house scripts written for IVision (BioVision Technologies, Exton, PA, USA). For brightfield colour imaging the imaging system comprises a Qimaging Micropublisher 3.3MP cooled colour CCD camera (Qimaging, Burnaby, BC), Zeiss Axioplan II fluorescence microscope with Plan-neofluar or Plan Apochromat objectives (Carl Zeiss, Welwyn Garden City, UK). Image capture was performed using in-house scripts written for IVision (BioVision Technologies, Exton, PA, USA).

Immunofluorescence stained sections were also imaged using a Nikon C1Si Confocal unit and Nikon inverted Eclipse TiE microscope and Nikon NIS Elements software (Nikon UK Limited, Surrey, KT2 5PR, UK). The Confocal unit allows the sections to be scanned and z-plane images to be formed.

CFU-F colonies were imaged by a Nikon AZ100 Macroscope (Nikon UK Limited, Surrey, KT2 5PR, UK) and a Qimaging Micropublisher camera. The imaging system comprises a Nikon AZ100 macroscope with 0.5x, 1x, 2x, 4x and 5x objectives, Intensilight 130W Hg light source and Nikon UV, G or B filter cubes (Nikon UK Ltd, Kingston-on-Thames, UK). For colour brightfield work a Qimaging Micropublisher 5 cooled colour camera was used (Qimaging, Burnaby, BC) and image capture was performed using in-house scripts written for IVision (BioVision Technologies, Exton, PA).

2.5 Electrophoresis
2.5.1 Gel electrophoresis of DNA samples

Genomic DNA was extracted using FlexiGene DNA Kit (#51204, QIAGEN Ltd., West Sussex, RH10 9NQ, UK). A 25 μl reaction mix was made of the following: 5 μl Genomic DNA, 2.5 μl 10x PCR Rxn Buffer (#P/N Y02028, Invitrogen Ltd, Paisley, PA4 9RF), 1.25 μl 50 mM MgCl₂ (#P/N Y02016, Invitrogen Ltd, Paisley, PA4 9RF), 0.5 μl Forward Primer, 0.5 μl Reverse Primer 1, 0.5 μl Reverse Primer 2,
2.5 μl 10x dNTPs (#201900, QIAGEN Ltd., West Sussex, RH10 9NQ, UK), 0.2 μl Taq DNA Polymerase (#10342-020, Invitrogen Ltd, Paisley, PA4 9RF), and 12.05 μl distilled water. The reaction mix was heated using a Peltier Thermal Cycler (PTC-225, MJ Research Inc., Canada, J3V 4PI) at the cycling conditions shown in Table 2.3. Primers were from Sigma (Sigma-Aldrich Company Ltd., Dorset, England), 100 μM stock solution and used at 1 in 8 dilution. Primer information is shown in Table 2.4.

DNA fragments were separated due to their size to assess whether tamoxifen successfully caused recombination of the Wt1 loxP sites using agarose gel electrophoresis. Recombined samples should show a band at ~521 BP, and control samples with no recombination will show a band at ~234 BP. A homozygous Wt1+/loxP sample and wildtype Wt1+/- sample were used as controls.

A 2% gel was used: 2.8 g Agarose (#300-300, BioGene Ltd., Kimbolton, PE28 ONJ, UK), 140 ml 1x TBE Buffer (See 8.1), microwave heated until dissolved and then 7 μl Ethidium Bromide added (Ethidium bromide 10 mg/ml, Electran® VWR International, Leicestershire, LE17 4XN). 5 μl 100 Base Pair DNA Ladder (#G2101, Promega UK, Southampton, SO16 7NS, UK) and 8 μl of DNA PCR mix plus Loading Dye (3 ml 100% Glycerol, 500 μl 20x TBE, 6.5 ml distilled water, and Bromophenol Blue for colouring) were loaded into individual wells of the gel which was then run for 50 minutes at 150 Volts with a PowerPac™ Basic Power Supply (Bio-Rad Laboratories Ltd., Hertfordshire, HP2 7DX). Gels were imaged on a BioDoc-It® LCD/LM-256 Imaging System (Ultra-Violet Products Ltd., Cambridge, CB4 1TG, UK).
Table 2.3 Cycling Conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pre Incubation</td>
<td>94°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>2. Amplification*</td>
<td>94°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td></td>
<td>58°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>3. Cooling</td>
<td>72°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

*Step 2 is repeated 35 times

Table 2.4. Primer Information for Gel Electrophoresis of DNA samples

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Direction</th>
<th>Primer Sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt1</td>
<td>Forward</td>
<td>TGGGTTCCAACCGTACCAAAGA</td>
</tr>
<tr>
<td>Wt1</td>
<td>Reverse 1</td>
<td>GGGCTTATCTCCTCCCATGT</td>
</tr>
<tr>
<td>Wt1</td>
<td>Reverse 2</td>
<td>GTACGCGCGAACACTGACTA</td>
</tr>
</tbody>
</table>

2.6 Gene Expression Analysis

2.6.1 RNA Extraction

2.6.1.1 RNA Isolation using TRIzol® Reagent

TRIzol® Reagent was used to isolate RNA from small quantities of tissue (50-100 mg) using a Polytron PT 3100 homogenizer. The TRIzol® Reagent protocol from the manufacturer’s manual was followed (#15596-026, Life Technologies Ltd, Paisley, PA4 9RF).

2.6.1.2 RNA Isolation and Purification using Qiagen RNeasy Mini Kit

Qiagen RNeasy Mini Kit (#74104, QIAGEN Ltd., West Sussex, RH10 9NQ, UK) was used to isolate RNA from cultured cells and to purify RNA isolated using the TRIzol® reagent. The manufacturer’s protocol was followed for Purification of Total RNA from Animal Cells using Spin Technology, and for RNA Cleanup, with optional on-column DNase Digestion step, all found in the RNeasy Mini Handbook.
2.6.1.3 RNA Isolation using Arcturus® PicoPure® RNA Isolation Kit

Arcturus® PicoPure® RNA Isolation Kit (#KIT0204, Applied Biosystems Arcturus Products, California, 92008, USA) was used to extract a high yield of RNA from small numbers of cells. The protocol provided by the manufacturer was followed.

2.6.1.4 RNA quality and quantity

RNA quantities extracted using the above methods were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE 19810, USA). The quality of the RNA was determined by Agnes Gallacher from the MRC HGU who performed Total RNA Analysis using an Agilent RNA 6000 Nano Chip and Agilent 2100 Bioanalyzer (both from Agilent Technologies UK Limited, Shropshire, SY7 8NR, UK).

2.6.2 cDNA Synthesis

cDNA was synthesised from RNA using a 40 µl reaction mix: 8 µl x5 First Strand Buffer, 0.4 µl 0.1 M DTT, 1 µl SuperScript III Reverse Transcriptase (all 3: #18080-044, Invitrogen Ltd, Paisley, PA4 9RF), 1 µl dNTPs (#201900, QIAGEN Ltd., West Sussex, RH10 9NQ, UK), 1 µl rRNasin RNase Inhibitor (#N251B, Promega, Madison, WI, USA), and 1.3 µl Random Primers (#C118A, Promega, Madison, WI, USA). The remaining volume was made up of 1000 ng RNA and RNase-free H₂O. The reaction mix was heated using a Peltier Thermal Cycler (PTC-225, MJ Research Inc., Canada, J3V 4PI) at 42°C for 2.5 hours.

2.6.3 Quantitative Realtime Polymerase Chain Reaction (qRT-PCR)

A 10 µl reaction mix was made for each sample of cDNA: 5 µl LightCycler 480 Probes Master (#04707494001, Roche Diagnostics Ltd., West Sussex, RH15 9RY), 0.1 µl Forward Primer, 0.1 µl Reverse Primer, 0.1 µl Probe, 2 µl cDNA, and 2.7 µl distilled H₂O. All primers were Custom Oligos from Sigma-Aldrich Company Ltd., Dorset, England, and all probes were from the Universal Probe Library (Roche
Diagnostics Ltd., West Sussex, RH15 9RY) and are detailed in Table 2.5. The 10 µl reaction mix for each sample was run on a clear Lightcycler® 384 well plate (Roche Diagnostics Corporation, Indianapolis, USA) in a LightCycler 480 II machine (Roche Diagnostics Ltd., West Sussex, RH15 9RY) under the cycling conditions shown in Table 2.6.

Table 2.5. Primer and probe information for each gene used in qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Direction</th>
<th>Primer Sequence (5'-'3')</th>
<th>Probe Number (UPL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt1</td>
<td>Forward</td>
<td>CAGGATGTCCCCCAATGC</td>
<td>33</td>
</tr>
<tr>
<td>Wt1</td>
<td>Reverse</td>
<td>TTGGTTCGGGATGTTAGG</td>
<td>33</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>Forward</td>
<td>GCTGCTGAGATGACAGGAA</td>
<td>76</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>Reverse</td>
<td>CCCTGTTTGAACATAAGGAAGC</td>
<td>76</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>Forward</td>
<td>GCTGCCGTCATTTTCTGC</td>
<td>3</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>Reverse</td>
<td>TCTCAGCTGGCCCGTCATC</td>
<td>3</td>
</tr>
<tr>
<td>18S</td>
<td>Forward</td>
<td>CGATTGGATGTTTAGTGAGG</td>
<td>81</td>
</tr>
<tr>
<td>18S</td>
<td>Reverse</td>
<td>AGTTGCACCGTCTTCTCAGC</td>
<td>81</td>
</tr>
</tbody>
</table>

Table 2.6. Cycling conditions for qRT-PCR

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pre Incubation</td>
<td>95°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>2. Amplification*</td>
<td>95°C</td>
<td>10 seconds</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>1 second</td>
</tr>
<tr>
<td>3. Cooling</td>
<td>40°C</td>
<td>30 seconds</td>
</tr>
</tbody>
</table>

*Step 2 is repeated 50 times
2.6.4 Custom qRT-PCR plates

RealTime ready Custom Panel 384 qRT-PCR plates (Figure 2.1) were designed and ordered using the RealTime ready Configurator (https://configurator.realtimeready.roche.com) (Roche Diagnostics Ltd., West Sussex, RH15 9RY). Each 384 well Custom plate was run in a LightCycler 480 II machine (Roche Diagnostics Ltd., West Sussex, RH15 9RY) under the cycling conditions shown in Table 2.7.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Wt1</td>
<td>Vegfa</td>
<td>Wtip</td>
<td>Ptgis</td>
<td>Fosl2</td>
<td>Egln3</td>
</tr>
<tr>
<td>B</td>
<td>Cited2</td>
<td>Mmp2</td>
<td>Smad3</td>
<td>Cryab</td>
<td>Vldlr</td>
<td>Sox4</td>
</tr>
<tr>
<td>C</td>
<td>Ddit4</td>
<td>Tgfb3</td>
<td>Cav1</td>
<td>Tgfb2</td>
<td>1190002H23Rik</td>
<td>Col1a1</td>
</tr>
<tr>
<td>D</td>
<td>Ctnnb1</td>
<td>Bcl9l</td>
<td>Snai1</td>
<td>Serpinh1</td>
<td>Arg1</td>
<td>Col3a1</td>
</tr>
<tr>
<td>E</td>
<td>Col5a1</td>
<td>Ddah1</td>
<td>Bcar1</td>
<td>Prkd2</td>
<td>Prkd1</td>
<td>Foxs1</td>
</tr>
<tr>
<td>F</td>
<td>Pdgfra</td>
<td>Foxc1</td>
<td>Fgfr2</td>
<td>Shox2</td>
<td>Tn</td>
<td>Sulf1</td>
</tr>
<tr>
<td>G</td>
<td>Mmp9</td>
<td>Stab1</td>
<td>Efnal</td>
<td>Ras</td>
<td>Thbs1</td>
<td>Hdac7</td>
</tr>
<tr>
<td>H</td>
<td>Adm</td>
<td>Hdac5</td>
<td>Sphk1</td>
<td>Creb3l2</td>
<td>Zbtb7a</td>
<td>Hspg2</td>
</tr>
<tr>
<td>I</td>
<td>Mmp13</td>
<td>Rarb</td>
<td>Prrx1</td>
<td>Prrx2</td>
<td>Zeb1</td>
<td>Gnas</td>
</tr>
<tr>
<td>J</td>
<td>Tnks</td>
<td>Csnk1d</td>
<td>Csnk1e</td>
<td>Dvl1</td>
<td>Ror2</td>
<td>Pparg</td>
</tr>
<tr>
<td>K</td>
<td>Clec7a</td>
<td>Tlr13</td>
<td>Csf1r</td>
<td>Fcgr1</td>
<td>Saa3</td>
<td>Il10</td>
</tr>
<tr>
<td>L</td>
<td>Ccl2</td>
<td>Ccl3</td>
<td>Ccl4</td>
<td>Ccl11</td>
<td>Fcgr3</td>
<td>Tnf</td>
</tr>
<tr>
<td>M</td>
<td>Ccr2</td>
<td>Tlr7</td>
<td>H2-Eb1</td>
<td>Irf7</td>
<td>Igf1</td>
<td>Sp7</td>
</tr>
<tr>
<td>N</td>
<td>Nfkbid</td>
<td>Pf4</td>
<td>Cxcl10</td>
<td>Dbp</td>
<td>Cdr2</td>
<td>Gapdh</td>
</tr>
<tr>
<td>O</td>
<td>Acta1</td>
<td>Angpt4</td>
<td>Itga11</td>
<td>Col12a1</td>
<td>Hif1a</td>
<td>Rn18s</td>
</tr>
<tr>
<td>P</td>
<td>Cdkn2b</td>
<td>Runx2</td>
<td>Ly86</td>
<td>Rpl13a</td>
<td>B2m</td>
<td>Actb</td>
</tr>
</tbody>
</table>

*Figure 2.1. The 96 genes replicated 4 times on each of the RealTime ready Custom Plates*
Table 2.7: Cycling conditions for qRT-PCR Custom Plates

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pre Incubation</td>
<td>95°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>2. Amplification*</td>
<td>95°C</td>
<td>10 seconds</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>1 second</td>
</tr>
<tr>
<td>3. Cooling</td>
<td>40°C</td>
<td>30 seconds</td>
</tr>
</tbody>
</table>

*Step 2 is repeated 45 times

2.7 Flow Cytometry: Fluorescence Activated Cell Sorting (FACS)

Fluorescence activated cell sorting was carried out by Elisabeth Freyer at MRC HGU using a BD FACS Aria™ II System (BD Biosciences, Oxford, OX4 4DQ, UK) equipped with 5 lasers and fluorescence detectors. Flow cytometry analysis was carried out using FlowJo Software Version 7.6.5 (http://www.flowjo.com, Tree Star, Inc., Ashland, 97520, USA). Bone marrow and enzymatically digested bone cells were isolated as previously described (See 2.3.1) and prepared as single cell suspensions in PBS/5%FCS. Cells were protected from the dark and incubated at 4°C for 15 minutes with the antibodies, with 5 minute PBS/5%FCS washes between stainings. Antibody information can be found in Table 2.8. Sorting gates were created using bone marrow and bone cells from nonstained wild type control mice. Isotype Controls and One Comp eBeads (#01-1111, eBioscience, Ltd., Hatfield, AL10 9NA, UK) were used as controls. Appendix 2 shows representative flow cytometry plots for gating strategies, dead cell removal, and singlet selection and Appendix 3 shows representative control quadrant selections.
Table 2.8. FACS Antibody dilution and wavelength information

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin Lineage Panel (CD3e, CD11b, CD45R, Ly-6G and Ly-6C, TER-119)</td>
<td>2μl of all 5</td>
<td>#559971, BD Pharmingen™, Oxford, OX4 4DQ, UK</td>
</tr>
<tr>
<td>Streptavidin PerCP-Cy5.5</td>
<td>1:600</td>
<td>#45-4317, eBioscience, Ltd., Hatfield, AL10 9NA, UK</td>
</tr>
<tr>
<td>CD31 PerCP-eFluor® 710</td>
<td>1:100</td>
<td>#46-0311, eBioscience, Ltd., Hatfield, AL10 9NA, UK</td>
</tr>
<tr>
<td>CD29 APC</td>
<td>1:40</td>
<td>#17-0291, eBioscience, Ltd., Hatfield, AL10 9NA, UK</td>
</tr>
<tr>
<td>CD105 PE</td>
<td>1:20</td>
<td>#12-1051, eBioscience, Ltd., Hatfield, AL10 9NA, UK</td>
</tr>
<tr>
<td>CD73 eFluor® 450</td>
<td>1:25</td>
<td>#48-0731, eBioscience, Ltd., Hatfield, AL10 9NA, UK</td>
</tr>
<tr>
<td>CD166 Biotinylated</td>
<td>1:50</td>
<td>#BAF1172, R&amp;D Systems Ltd., Abingdon, OX14 3NB</td>
</tr>
<tr>
<td>Sca-1 PB</td>
<td>1:150</td>
<td>#108120, BioLegend, London, NW5 1LB, UK</td>
</tr>
<tr>
<td>CD146 APC</td>
<td>1:100</td>
<td>#130-098, Miltenyi Biotec Ltd., Surrey, GU24 9DR</td>
</tr>
<tr>
<td>CD29 PB</td>
<td>1:300</td>
<td>#102224, BioLegend, London, NW5 1LB, UK</td>
</tr>
</tbody>
</table>

2.8 Gene Expression Microarrays

2.8.1 Microarray sample preparation – cRNA amplification

Total bone marrow and total bone were prepared from control and mutant WGER mice (See 2.3.1) as well as stained and FACS sorted for a non-haematopoietic population using Lineage panel and CD31 antibodies (See Appendix 4&5 for a schematic diagram). RNA was extracted from GFP+ and GFP- cells from total and haematopoietic lineage negative, control and mutant, bone marrow and bone using the TRizol® Reagent (Invitrogen Ltd, Paisley, PA4 9RF, UK) procedure outlined previously. cRNA, prepared from RNA (RIN value >7), was amplified according to the Illumina® TotalPrep RNA Amplification Kit Instruction Manual (Life Technologies Ltd., Paisley, PA4 9RF, UK).

2.8.2 Gene Expression

cRNA yield and quality was assessed by Louise Evenden and Audrey Duncan at the Edinburgh Wellcome Trust Clinical Research Facility. Gene Expression was performed using the Illumina HT12 v4 Expression BeadChip and Illumina Whole-Genome Gene Expression Direct Hybridisation Assay (WGGX) (Illumina UK,
Essex, CB10 1XL, UK). This consists of a hybridisation step followed by washing and staining of the bead chip prior to imaging. For each of the samples, 5 µl of 150 ng/µl cRNA (quantified using the Agilent 2100 Bioanalyser) was used. The arrays were imaged on the Illumina HiScanSQ platform (Illumina UK, Essex, CB10 1XL, UK) and genotypes were called automatically using GenomeStudio Analysis Software Version 2011.1 (Illumina UK, Essex, CB10 1XL, UK).

2.8.3 Gene Expression Analysis

Illumina Gene Expression data was analysed by Graeme Grimes from MRC HGU. To determine correlations in the expression data, Pearson correlation coefficients were calculated with Microsoft Excel (Microsoft UK, Berkshire, RG6 1WG, UK) and hierarchical clustering of each subset was performed using R (Version 2.9.0 http://www.r-project.org/). Analysis was carried out using the R beadarray (Dunning et al., 2007) and Limma Version 3.10.2 Software (Smyth, 2004). In order to compare the data from multiple arrays Quantile normalisation was applied as well as a linear model for the expression data of each gene. Statistically differentially expressed genes were determined using the linear model results with an applied Bayes moderated t test. A Benjamini and Hochberg false discovery rate was used as a multiple testing control with a p-value of < 0.05 (Analysis similar to that seen in Reijns et al., 2012).

The Gene Ontology Enrichment Analysis and Visualization tool (GOrrilla cbl-gorilla.cs.technion.ac.il) was used to identify enriched GO terms from a ranked list of differentially expressed genes. The Database for Annotation, Visualization and Integrated Discovery Version6.7 (http://david.abcc.ncifcrf.gov/) was used to identify enriched biological themes, enriched functional-related gene groups, and pathway maps.

2.9 Statistical Analysis

The data is expressed as a mean value (± Standard Error), with p values determined using a Two-Tailed t test. Statistically significant is defined as p<0.05.
Chapter 3

Characterisation of Wt1\(^+\) population in bone and bone marrow
3.1 Introduction

There is little knowledge of the expression pattern or role that \textit{Wt1} may play in bone formation and maintenance. However, as previously discussed it seems to be a potential candidate due to its role in maintaining various adult tissues including bone (Chau \textit{et al.}, 2011). So far in the bone marrow \textit{Wt1} expression has only been detected in a small percentage (1.2\%) of CD34\(^+\) haematopoietic progenitor cells (Hosen \textit{et al.}, 2002). Therefore, I aimed to isolate \textit{Wt1} expressing cells from the entire bone marrow population and investigate what these are. The bone marrow is the main source of MSCs and they are known to be located in the endosteal region which lines the bone (Muguruma \textit{et al.}, 2006). Therefore, a second aim was to elucidate whether \textit{Wt1\(^+\)} cells could be related to bone marrow derived or bone derived MSCs and/or their progeny. \textit{Wt1} is expressed in a small percentage of marrow cells, and similarly MSCs only make up between 0.01\% and 0.001\% of the marrow (Pittenger \textit{et al.}, 1999) making these two populations hard to isolate, define, and relate to each other.

In summary, this chapter shows that \textit{Wt1} expressing cells are found in the marrow, and for the first time in the bone. The \textit{Wt1} expressing GFP population overlaps with MSC markers, but more so with the markers for osteoblast progenitors.

3.2 Characterising the GFP positive population \textit{in vivo} and \textit{in vitro}

To isolate and characterise the \textit{Wt1} positive cells in the marrow and bone, fluorescence activated cell sorting (FACS) was used. Using a \textit{Wt1}-GFP mouse with GFP knock-in reporter at the \textit{Wt1} locus, GFP cells could be isolated using FACS. The model was validated using qRT-PCR to quantify \textit{Wt1} expression, showing that the GFP population is positive for endogenous \textit{Wt1} (Figure 3.1). Flow cytometry gates were set using GFP negative cells, this controlled for autofluorescence (Appendix 2 for representative plots). Sorting the same population twice established the sort purity as 98\% pure.
Here we show that GFP, and therefore Wt1 expressing, cells are found in both the marrow cells, and for the first time in the bone. This was then quantified by qRT-PCR to show that GFP positive cells are indeed expressing Wt1 in both marrow and bone in vivo (Figure 3.1). In vitro, on the other hand, hypoxia can stimulate the expression of Wt1 through transcriptional activation of HIF1 (Wagner et al., 2003). Hence, I set out to look in vitro at the effect of hypoxia on the cultured GFP population. The majority of bone marrow studies are carried out under standard culture incubator conditions of 5% CO₂ and 95% air (20% oxygen) (For example: Sun et al., 2003; Peister et al., 2004). As Fehrer et al. (2007) previously stated with regard to bone marrow, 3% O₂ (i.e. hypoxic culture conditions) is more appropriate physiologically in the context of this study. Interestingly hypoxia caused a dramatic increase in the percentage of cells which are GFP positive in both marrow and bone cells (Figure 3.2). These data may also concur with Wagner et al. (2003) who showed that Wt1 is stimulated by hypoxia, specifically via transcriptional activation of HIF-1. Wagner et al. (2003) showed the upregulation of Wt1 mRNA and protein in the kidneys and heart, however this response is a novel finding for the bone and bone marrow. Further studies are needed to verify this and also to determine whether this increase in GFP percentage is due to increased proliferation of this population or an increased expression in previously negative cells.
Figure 3.1. In Vivo Wt1 mRNA Expression. Mean Wt1 mRNA expression (±SEM) is highest in GFP positive sorted cells compared to the GFP negative cells from Wt1-GFP model, and NIH3T3 negative control fibroblasts, in both marrow and bone (* = p<0.05) (n=3).

Figure 3.2. Percentage of GFP+ cells found in MSC cultures increases under hypoxic conditions. A. A representative flow cytometry scatter plot showing GFP positive cells (GFP pos) in marrow MSCs cultured under normoxia (20% O₂). B. A representative flow cytometry scatter plot showing GFP positive cells (GFP pos) in marrow MSCs cultured under hypoxia (3% O₂). C. The mean number (±SEM) of GFP+ cells as a percentage of the total bone marrow population cultured under MSC favouring conditions, in either normoxia or hypoxia. (*** = p<0.001)(n=12). D. The mean number (±SEM) of GFP+ cells as a percentage of the total bone population cultured under MSC favouring conditions, in either normoxia or hypoxia. (** = p<0.01)(n=13).
To characterise the functional role of \textit{Wt1} in the \textit{Wt1}-GFP positive population of cells, both under normoxia and hypoxia, the effect of \textit{Wt1} deletion was studied. This was carried out using the CAGG-CreER\textsuperscript{TM};\textit{Wt1}\textsuperscript{loxP/GFP} (WGER) mouse with a tamoxifen inducible \textit{Wt1} deletion. The CAGG promoter ensures that the Cre is ubiquitously expressed. Tamoxifen has no effect on the Cre\textsuperscript{−} cells (or controls), but on the Cre\textsuperscript{+} cells (or mutants) the CreER recombinase is activated and recombines the \textit{loxP} sites, excising the gene. In this model, as previously demonstrated, the GFP sorted cells express \textit{Wt1} and following the deletion of \textit{Wt1} the cells continue to express GFP. Figure 3.3 verifies that \textit{in vitro} \textit{Wt1} deletion is effective, i.e. 4-OH tamoxifen causes Cre mediated \textit{loxP} recombination, and \textit{Wt1} expression is lost.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig3.png}
\caption{\textbf{In Vitro Wt1 mRNA expression.} 4-OH Tamoxifen effectively deletes \textit{Wt1} in mutant bone cell cultures as shown by the loss of mean (±SEM) \textit{Wt1} mRNA expression under \textbf{A} normoxia (p<0.05) (n=3) and \textbf{B} hypoxia (p=0.1) (n=3).}
\end{figure}
3.3 Relating the \textit{Wt1}-expressing GFP population to an MSC population

3.3.1. \textit{In Vivo}

Marrow and bone cells from Cre$^{-}$ control WGER mice were stained with a lineage panel (CD3e, CD11b, CD45R, Ly-6G and Ly-6C, TER-119) and CD31 endothelial marker to identify the haematopoietic and endothelial fraction. The cells were also stained with three MSC cell surface markers (CD29, CD73, and CD105) in order to relate the non-haematopoietic (i.e. Lin$^{-}$CD31$^{-}$) MSC population (Triple$^{+}$) to the GFP positive population (GFP$^{+}$).

The first interesting finding is that GFP (and therefore \textit{Wt1} expressing) cells are found in both the marrow cells, and for the first time in the bone. This novel finding is important as prior to this \textit{Wt1} expression was not identified in many adult tissues, and was only shown to be expressed in 1.2% of adult human bone marrow, specifically the CD34$^{+}$ haematopoietic stem and progenitor cell population (Hosen \textit{et al.}, 2002).

The \textit{in vivo} results show 3.03% of bone marrow is GFP$^{+}$ (Figure 3.6) and 0.91% of bone is GFP$^{+}$ (Figure 3.7). Figure 3.6 shows that 64.5% of our GFP bone marrow population is haematopoietic meaning of the total haematopoietic compartment 1.95% are GFP$^{+}$. Hosen \textit{et al.}, (2002) show a small percentage (1.2%) of HSCs are \textit{Wt1}$^{+}$ suggesting that the remaining GFP$^{+}$ cells identified here are found in the remaining haematopoietic population. Encouragingly, 7.91% of the total GFP population in the bone marrow are non-haematopoietic MSCs (i.e. Lin$^{-}$CD31$^{-}$Triple$^{+}$) which shows that the GFP population is overlapping with the MSC population. This 7.91% of GFP$^{+}$ cells only accounts for 0.24% of the total marrow population. The GFP$^{+}$ and MSC cell populations also overlap in the bone but at a much smaller proportion (0.5% of the GFP population is Triple$^{+}$).

The adult phenotype reduction of MSC derived bone, fat, and osteoblasts seen following \textit{Wt1} deletion makes this GFP/MSC overlap very interesting. To look at what functional effect \textit{Wt1} has on these cells WGER mice were injected intraperitoneally for 5 days with tamoxifen. This resulted in \textit{Wt1} deletion \textit{in vivo},
followed by the analysis of bone and marrow cells at day 10. Following Wt1 deletion there was no significant difference in the various cell populations which make up the total population for either bone marrow (Figure 3.4) or bone (Figure 3.5). However, as a proportion of the total GFP population, there was a reduction in the Lin’CD31’ population and significant reduction (p<0.05) in the MSC Triple+ population in the Wt1 knockout marrow cells (Figure 3.6). The same trend is also seen in the bone cells, with a reduction in both populations, non-haematopoietic and triple marker positive, following the Wt1 knockout (Figure 3.7).

It is interesting that, for both bone and marrow, as a proportion of the total cell population the Lin’CD31’Triple+GFP’ proportion is not altered by Wt1 deletion, but as a proportion of the GFP population, it is. After seeing such a dramatic bone and fat loss phenotype in the adult ubiquitous Wt1 deletion (Chau et al., 2011) it is intriguing that there is such a small effect seen here. Indeed, the bone, fat, and osteoblast reductions seen after the ubiquitous deletion were the reason to investigate the GFP population in relation to the MSCs. This is discussed further after this experiment was repeated in vitro.
Figure 3.4. In Vivo Deletion of Wt1 has no effect on the bone marrow sub populations. The mean (±SEM) percentage of total cells shows no change when Wt1 is deleted in the following subpopulations: Haematopoietic cells (Lin<sup>+</sup>CD31<sup>+</sup>) (p=0.84), Non-haematopoietic cells negative for any of the triple MSC markers (Lin<sup>-</sup>CD31<sup>-</sup> NonTriple`) (p=0.98), Non-haematopoietic cells positive for all three MSC markers but negative for GFP (Lin<sup>-</sup>CD31<sup>-</sup> Triple`GFP`) (p=0.12), and Non-haematopoietic cells positive for all three MSC markers and GFP (Lin<sup>-</sup>CD31<sup>-</sup>Triple`GFP`) (p=0.13) (control n=4, mutant n=3).

Figure 3.5. In Vivo Deletion of Wt1 has no effect on the bone sub populations. The mean (±SEM) percentage of total cells shows no change when Wt1 is deleted in the following bone subpopulations: Haematopoietic cells (Lin<sup>+</sup>CD31<sup>+</sup>) (p=0.19), Non-haematopoietic cells negative for any of the triple MSC markers (Lin<sup>-</sup>CD31<sup>-</sup> NonTriple`) (p=0.18), Non-haematopoietic cells positive for all three MSC markers but negative for GFP (Lin<sup>-</sup>CD31<sup>-</sup>Triple`GFP`) (p=0.60), and Non-haematopoietic cells positive for all three MSC markers and GFP (Lin<sup>-</sup>CD31<sup>-</sup>Triple`GFP`) (p=0.47) (control n=4, mutant n=3).
Figure 3.6. In Vivo deletion of Wt1 has an effect on the GFP subpopulations in the bone marrow. A. The deletion of Wt1 has no effect on the mean (±SEM) percentage of GFP cells in total bone marrow. B. However, it does have an effect on the mean (±SEM) subpopulation proportions. There is a reduction in total non-haematopoietic cells (p=0.13), a reduction in non-haematopoietic negative for any of the 3 MSC markers (Lin-CD31- NonTriple+) (p=0.18), and a significant reduction in the non-haematopoietic cells positive for the 3 MSC markers (Lin+ CD31+ Triple+) (p<0.05) (control n=4, mutant n=3).
Figure 3.7. In Vivo deletion of Wt1 has an effect on the GFP subpopulations in bone. 
A. The deletion of Wt1 has little effect on the mean (±SEM) percentage of GFP cells in total bone. B. However, it seems to have a greater effect on the mean (±SEM) subpopulation proportions. There is a reduction in total non-haematopoietic cells (p=0.12), a reduction in non-haematopoietic negative for any of the 3 MSC markers (Lin-CD31-NonTriple⁺) (p=0.12), and a reduction in the non-haematopoietic cells positive for the 3 MSC markers (Lin⁺CD31⁺Triple⁺) (p=0.09) (control n=4, mutant n=3).
3.3.2. *In Vitro*

It is possible that any *Wt1* deletion effects caused may be too subtle to be observed *in vivo*, or effects would not be observed within 10 days, the time at which the animals were culled. Therefore the experiment was repeated *in vitro*. Bone and marrow was again harvested from Cre control WGER mice and cultured under normoxia (20% Oxygen) and hypoxia (3% Oxygen). Again GFP cells were seen in bone and marrow, and in both cases the percentage increased under hypoxia. There was also the overlap in GFP and MSC populations. *In vitro*, however, the percentage was greater in the bone rather than marrow; 11.52% of the GFP population overlaps with the Lin£¬CD31£¬Triple£± cells (i.e. MSC population) when cultured under normoxia (Figure 3.10). This overlap was also seen when bone was cultured under hypoxia, but at slightly reduced values. In the marrow, the MSC and GFP populations also overlap, but at a smaller percentage (Figure 3.9). Compared with *in vivo* data, cells cultured *in vitro* seem to have a higher percentage of GFP£± cells in bone and marrow. *In vivo* marrow is 3.03% GFP£± compared to 7.33% of *in vitro* hypoxia cultured marrow. The same was seen for bone; *in vivo* is 0.91% GFP£± compared to 7.14% of *in vitro* hypoxia cultured bone. On the other hand, when looking at the proportion of cells which are Lin£¬CD31£¬Triple£±GFP£± values are the same in *in vivo* marrow and *in vitro* marrow cultured under hypoxia; 0.24%. However, in the bone the value *in vivo* is 0.003% and is increased when cultured *in vitro* under both normoxia (0.12%) and hypoxia (0.43%).

To again investigate the functional effects of *Wt1*, bone and marrow cells from WGER mice were cultured with 4-OH tamoxifen to cause the tamoxifen mediated *Wt1* deletion *in vitro*. These cells were cultured under normoxic and hypoxic conditions. Again, *Wt1* deletion showed no effect on the subpopulations of bone or marrow, cultured under normoxia or hypoxia (Figure 3.8). When analysing the subpopulations within the GFP cells, despite not being affected by *Wt1* deletion in the bone marrow, they are affected by hypoxia. The total bone marrow GFP proportion increases with hypoxia (Figure 3.9A) which is not wholly unexpected as we have shown this previously in Figure 3.2. However, hypoxia also causes an increase in Lin£¬CD31£¬Triple£± cells (p=0.06), a significant increase in Lin£¬CD31£¬
NonTriple⁺ cells (p<0.05) and a significant decrease in Lin⁺CD31⁺ cells (p<0.05) (Figure 3.9B). This shows that hypoxia can affect the proportional make-up of the GFP population in the bone marrow.

When looked at in the bone, the deletion of Wt1 has an unexpected effect on the GFP population with it increasing significantly in the mutant under both normoxia and hypoxia (p<0.05 for both). However, unlike the trend seen in the bone marrow, hypoxia does not seem to have a significant effect on the bone total GFP population despite it showing a similar trend increase (p=0.13) (Figure 3.10A). Unlike the total GFP, the GFP subpopulations were not affected by Wt1 deletion in the bone, but as seen in the bone marrow, were affected by oxygen culture levels. Bone showed the same trends as the marrow with a significant increase seen in the hypoxic Lin⁻CD31⁻ NonTriple⁺ population (p<0.01) and a significant decrease in the hypoxic Lin⁺CD31⁺ population (p<0.05) (Figure 3.10B). Again this shows that hypoxia is affecting the make-up of the GFP population in the bone, but also that the loss of Wt1 expression causes an increase in the total GFP population.
Figure 3.8. In Vitro Wt1 Deletion has no effect on bone marrow or bone subpopulations cultured under normoxia (n) or hypoxia (h). **A.** Mean (±SEM) percentage of total bone marrow shows no change following Wt1 deletion in the following subpopulations: Haematopoietic (Lin-CD31+) (n:p=0.38, h:p=0.67), Non-haematopoietic, negative for any triple MSC markers (Lin-CD31-NonTriple+) (n:p=0.46, h:p=0.41), Non-haematopoietic positive for three MSC markers, negative for GFP (Lin-CD31+Triple-GFP-) (n:p=0.16, h:p=0.43), and Non-haematopoietic positive for all three MSC markers and GFP (Lin-CD31+Triple-GFP+) (n:p=0.77, h:p=0.32) (control n=7, mutant n=3). **B.** The mean (±SEM) percentage of total bone shows no change following Wt1 deletion in the following subpopulations: Lin-CD31+ (n:p=0.518, h:p=0.15), Lin CD31 NonTriple+ (n:p=0.30, h:p=0.30), Lin CD31 Triple-GFP (n:p=0.09, h:p=0.39), and Lin CD31 Triple-GFP+ (n:p=0.16, h:p=0.06) (control n=8, mutant n=3).
**Figure 3.9. In Vitro Wt1 Deletion has no effect on bone marrow subpopulations, however hypoxia does.**

**A.** Wt1 deletion does not affect the mean (±SEM) GFP proportion of total bone marrow (n:p=0.42, h:p=0.76). However, the mean (±SEM) GFP proportion is affected by hypoxia increasing significantly in both control (p<0.01) and mutant (p<0.05) bone cells. **B.** Likewise, the GFP subpopulations are not affected by Wt1 deletion (Lin\(^{-}\)CD31\(^{-}\) Triple\(^{+}\) n:p=0.63, h:p=0.45; Lin\(^{-}\)CD31\(^{-}\) NonTriple\(^{+}\) n:p=0.60, h:p=0.51; Lin\(^{+}\)CD31\(^{-}\) Triple\(^{+}\) n:p=0.98, h:p=0.08), but hypoxia causes an increase in Lin\(^{-}\)CD31\(^{-}\) Triple\(^{+}\) (p=0.06), significant increase in Lin\(^{-}\)CD31\(^{-}\) NonTriple\(^{+}\) (p<0.05) and significant decrease in Lin\(^{+}\)CD31\(^{-}\) (p<0.05) (control n=8, mutant n=3).
**Figure 3.10. In Vitro Wt1 deletion has an effect on the bone GFP population but not on subpopulations, however hypoxia does.**

**A.** Wt1 deletion causes an increase in the mean (±SEM) GFP proportion of total bone (n&h:p<0.05).

**B.** Unlike the total GFP, the GFP subpopulations are not significantly affected by Wt1 deletion (Lin−CD31− n:p=0.41, h:p=0.08; Lin−CD31− NonTriple n:p=0.44, h:p=0.10; Lin CD31 Triple n:p=0.41, h:p=0.46). However, hypoxia does causes a significant increase in Lin−CD31− NonTriple (p<0.01) and significant decrease in Lin+CD31+ (p<0.05) (control n=8, mutant n=3).
3.4 Relating the GFP population to osteoblast progenitor markers

Nakamura et al. (2010) have characterised a subpopulation of non-haematopoietic and non-endothelial (CD45^−CD31^−Ter119^−) adult bone marrow cells by their expression of Sca-1 and CD166 cell surface markers. The CD166^+Sca-1^− and CD166^+Sca-1^− populations were enriched for osteoblasts and had osteogenic potential. CD166^+Sca-1^− cells showed some lipid accumulation indicating adipogenesis, but CD166^+Sca-1^− did not. The CD166^+Sca-1^− cells also showed evidence of chondrocytes and chondrogenesis, but less so in the CD166^+Sca-1^− population. The CD166^+Sca-1^+ population was not characterised by their study and the CD166^+Sca-1^+ cells were enriched for immature mesenchymal cells capable of osteogenesis and adipogenesis. These data are summarised in Figure 3.11.

\[\text{Figure 3.11. Representative flow cytometry plot summarising the 4 different quartiles described by Nakamura et al. (2010).}\]
3.4.1. In Vivo

Bone and marrow were obtained and cells were stained for the lineage panel markers (CD3ε, CD11b, CD45R, Ly-6G and Ly-6C, TER-119), CD31 endothelial marker, CD166 and Sca-1 markers. This was to assess the in vivo osteogenic potential of these non-haematopoietic cells and to identify where the GFP population was distributed relative to these markers. Appendix 2 and 3 show flow cytometry plots which established the quadrants and gates.

The Lin'CD31' marrow cells i.e. non-haematopoietic cells were mainly found in the CD166'Sca-1' and CD166'Sca-1-' populations, which as previously described are the populations enriched for osteoblasts and with osteogenic potential (Figure 3.12 A). This is also the case for the Lin'CD31' bone cells (Figure 3.13 A). Interestingly, when looking at the distribution of the Lin'CD31' GFP population, the same trend was seen; GFP cells were mainly found in the CD166'Sca-1' and CD166'Sca-1-' quartiles, for both marrow (Figure 3.12 D) and bone (Figure 3.13 D). However, in the marrow there were also a significant proportion of cells in the CD166'+Sca-1' quadrant, which is currently an uncharacterised population. Nakamura et al. (2010) also saw the highest proportion of non-haematopoietic bone and marrow cells in the CD166'Sca-1' quadrant. The second highest proportion in the bone marrow seen by Nakamura et al. (2010) was in the CD166'+Sca-1' quadrant, as also seen here. However, in the bone Nakamura et al. (2010) saw the second highest proportion in the CD166'Sca-1' quadrant. In the present study, this was the quartile with the least cells. Despite the GFP cells being mainly in the CD166'Sca-1' and CD166'Sca-1' quartiles, the proportion of the quartile made up by the GFP+ cells was highest in the uncharacterised CD166'+Sca-1' population (Marrow: Figure 3.12 C, Bone: Figure 3.13 C). 7.10% of the CD166'+Sca-1' quartile was GFP+ in the marrow and 2.36% was GFP+ in the bone. (Marrow: CD166'+Sca-1' 2.79%, CD166'Sca-1' 5.17%, CD166'Sca-1' 2.41%; Bone: CD166'+Sca-1' 1.14%, CD166'Sca-1' 0.63%, CD166'Sca-1' 0.62%).

To assess the functional role of Wt1 on the osteogenic potential Wt1 deletion was carried out in vivo via the intraperitoneal injection of tamoxifen. Bone and marrow
were obtained and cells were again stained for the lineage panel markers (CD3e, CD11b, CD45R, Ly-6G and Ly-6C, TER-119), CD31 endothelial marker, and then stained with CD166 and Sca-1 markers.

The deletion of Wt1 seems to have no significant effect, however a trend can be seen in both total and GFP cells for marrow and bone. Following Wt1 deletion, the CD166\(^+\)Sca-1\(^-\) population decreased and the CD166\(^-\)Sca-1\(^-\) population increased, suggesting a shift of cells between quartiles and a change in their adipogenic and chondrogenic abilities (Nakamura et al., 2010) (Marrow: Figure 3.12 A&D, Bone: Figure 3.13 A&D). This trend was observed in Lin\(^-\)CD31\(^-\) marrow (decrease p=0.23 and increase p=0.13), Lin\(^-\)CD31\(^-\) bone (decrease p=0.07 and increase p=0.06), Lin\(^-\)CD31\(^-\)GFP\(^+\) marrow (decrease p=0.27) and Lin\(^-\)CD31\(^-\)GFP\(^+\) bone (decrease p=0.33 and increase p=0.23) (Marrow: Figure 3.12 A&D, Bone: Figure 3.13 A&D).

The deletion of Wt1 has a negative effect on the proportion of GFP positive cells in each quartile suggesting that although the loss of Wt1 expression does not significantly alter the distribution of GFP\(^+\) cells it does alter the GFP population size and therefore the percentage in each quartile. The proportion of GFP\(^+\) cells was decreased in marrow (CD166\(^+\)Sca-1\(^-\) p=0.38, CD166\(^+\)Sca-1\(^+\) p=0.58, CD166\(^-\)Sca-1\(^+\) p=0.86, CD166\(^+\)Sca-1\(^-\) p=0.06) (Figure 3.12 C) and bone (CD166\(^+\)Sca-1\(^-\) p=0.06, CD166\(^+\)Sca-1\(^-\) p=0.04, CD166\(^+\)Sca-1\(^+\) p=0.08, CD166\(^+\)Sca-1\(^-\) p=0.08) (Figure 3.13 C), with the reduction in the CD166\(^+\)Sca-1\(^-\) quartile of the bone being significant (p<0.05).

3.4.2. In Vitro

The osteogenic capacity of bone and marrow cells was also investigated in vitro, cultured under either normoxic or hypoxic conditions for one week. The distribution of cells differed greatly to what was seen in vivo. The distribution of Lin\(^-\)CD31\(^-\) marrow cells showed the majority being in the CD166\(^+\)Sca-1\(^-\) quartile, for both normoxia and hypoxia (Figure 3.14 A & B respectively). The remaining population was then found in the CD166\(^+\)Sca-1\(^-\) population and the uncharacterised CD166\(^+\)Sca-1\(^+\) population, unlike in vivo. The Lin\(^-\)CD31\(^+\)GFP\(^+\) marrow cells, however, were mainly found in the uncharacterised CD166\(^+\)Sca-1\(^+\) population, under both normoxia
and hypoxia (Figure 3.14 E & F respectively). This was also seen in the bone. Lin−CD31− and LinCD31 GFP+ cultured in either normoxic or hypoxic conditions were all primarily found in the CD166+Sca-1+ quartile (Figure 3.15 A, B, E & F). This shows that the cell marker profile of both marrow and bone changes once cultured in vitro. As with the in vivo results, the GFP proportion of each quartile was again highest in the CD166+Sca-1+ population (Marrow: Figure 3.14 C & D, Bone: Figure 3.15 C).

A common result between bone, marrow, in vivo, and in vitro, Lin’CD31’, and GFP+, was that the quadrant with the least proportion of cells was always the CD166−Sca-1+; the immature mesenchymal cell population. This means that the majority of the GFP population either contains osteoblasts or has osteogenic potential, rather than immature mesenchymal cells.

Deletion of Wt1 was carried out to assess the functional role of this gene. Unlike the in vivo study, the deletion of Wt1 after 1 week of culture, does not affect the distribution of cells in any uniform way. Some of the quartiles show a reduction in the GFP proportion following Wt1 deletion but not all and not significantly. It is likely that this is due to the small sample size (n=2). A reduction was seen in the following quartiles: normoxic marrow CD166+Sca-1− p=0.27, CD166+Sca-1+ p=0.37, CD166−Sca-1+ p=0.82, hypoxia marrow CD166+Sca-1− p=0.37, CD166−Sca-1+ p=0.24, normoxic bone CD166+Sca-1− p=0.86, CD166−Sca-1− p=0.53 and hypoxic bone CD166+Sca-1− p=0.96, CD166−Sca-1+ p=0.17, CD166−Sca-1+ p=0.49, CD166−Sca-1− p=0.81.

The ubiquitous deletion of Wt1 in adults resulted in reduced osteoblast synthesis due to impaired differentiation abilities (Chau et al., 2011). Despite Wt1 deletion having little effect on marrow and bone non-haematopoietic cell distribution across the quadrants, either in vivo or in vitro, the functional capabilities of these cells have not been tested and could be impaired; therefore differentiation needs to be investigated.
Figure 3.12. The in vivo osteogenic distribution of Lin\(^{-}\)CD31\(^{+}\) marrow and GFP cells. A. The mean (±SEM) percentage of Lin\(^{-}\)CD31\(^{+}\) marrow cells in each quartile for control and mutant. B. A representative flow cytometry plot of the Lin\(^{-}\)CD31\(^{+}\) marrow distribution. C. The mean (±SEM) percentage of marrow cells which express GFP in each quartile, for control and mutant. D. The mean (±SEM) percentage of total GFP cells in each quartile, for control and mutant. E. A representative flow cytometry plot of the total GFP cell distribution. (control \(n=4\), mutant \(n=3\)).
Figure 3.13. The in vivo osteogenic distribution of Lin^CD31^ bone and GFP cells. **A.** The mean (±SEM) percentage of Lin^CD31^ bone cells in each quartile for control and mutant. **B.** A representative flow cytometry plot of the Lin^CD31^ bone distribution. **C.** The mean (±SEM) percentage of bone cells which express GFP in each quartile, for control and mutant. Wt1 deletion has a significant effect on all populations (p<0.05). **D.** Mean (±SEM) percentage of total GFP cells in each quartile, for control and mutant. **E.** A representative flow cytometry plot of the total GFP cell distribution. (control n=4, mutant n=3).
Figure 3.14. The in vitro osteogenic distribution of total marrow and GFP cells. The mean (±SEM) percentage of total marrow cells in each quartile for control and mutant cultured under A, normoxia and B, hypoxia. The mean (±SEM) percentage of marrow cells which express GFP in each quartile, for control and mutant, cultured under C, normoxia and D, hypoxia. The mean (±SEM) percentage of total GFP cells in each quartile, for control and mutant, cultured under E, normoxia and F, hypoxia. (n=2).
Figure 3.15. The in vitro osteogenic distribution of total bone and GFP cells. The mean (±SEM) percentage of total bone cells in each quartile for control and mutant cultured under A. normoxia and B. hypoxia. The mean (±SEM) percentage of bone cells which express GFP in each quartile, for control and mutant, cultured under C. normoxia and D. hypoxia. The mean (±SEM) percentage of total GFP cells in each quartile, for control and mutant, cultured under E. normoxia and F. hypoxia. (n=2).
3.5 Discussion

The results from this chapter have shown that $Wt1$ expressing cells can be isolated from the bone and marrow using a GFP knock in and flow cytometry. Interestingly, hypoxia has a huge effect on the GFP ($Wt1$ positive) population. As discussed previously 3% Oxygen more closely mimics physiological conditions than standard culture conditions (i.e. 20% Oxygen). Here I show that in vivo marrow cells are 3.03% GFP positive which is similar to the 2.54% of cells cultured in vitro under normoxia. Both are significantly less than the 13.67% of in vitro hypoxia cultured marrow cells. The same trend is seen in the bone; 0.91% of bone cells are GFP$^+$ in vivo, and in vitro 2.60% of cells cultured under normoxia are GFP$^+$ compared to 8.01% in hypoxia. These values would suggest that the in vivo data are more closely matched to the in vitro normoxic results rather than hypoxic. However, we need to bear in mind that the in vitro values are taken from an adherent population of cells which have been cultured for 7 days, making them less comparable with the in vivo cells.

One explanation for the increased proportion of GFP under hypoxia is an increase in proliferation. Assays have been carried out unsuccessfully on these cells due to fixing agents which abolish the GFP signal. An alternative avenue would be to culture GFP negative cells under hypoxia to determine whether hypoxia is inducing $Wt1$ expression and therefore GFP. This is relatively likely as Wagner et al. (2003) has already shown HIF-1 targets $Wt1$, but due to time restriction I was unable to follow it up. As Wagner et al. (2003) have only shown this in the kidney and heart, identifying it in the bone and marrow would be novel and therefore an area of future interest and research.

As well as isolating $Wt1$ expressing cells, an MSC population was identified using three cell surface markers (CD29, CD73, and CD105). These ‘MSCs’ were seen in fresh bone and marrow as well as cultured. In vivo, the non-haematopoietic portion of the MSCs were isolated and found to express GFP in the bone marrow (3.23%) and bone (0.99%). These non-haematopoietic GFP$^+$ MSCs only make up a small percentage of the total GFP population in the bone marrow (7.91%) and bone.
This is also seen in vitro; GFP is expressed in the non-haematopoietic MSCs in the normoxic marrow (0.82%), hypoxic marrow (10.42%), normoxic bone (3.7%), and hypoxic bone (10.24%). Yet of the overall GFP population, non-haematopoietic GFP⁺ MSCs make up a small percentage: normoxic marrow (1.12%), hypoxic marrow (2.91%), normoxic bone (11.51%), and hypoxic bone (8.05%). This begs the question ‘what are the remaining GFP positive cells?’.

One explanation is due to the poor definition of an MSC and notorious difficulty found in isolating them from mice. The literature offers a multitude of markers in various combinations for the isolation of MSCs, most of which are also markers and characteristic to other cell types (Silva et al., 2003). It may be, therefore, that these markers are too stringent and restrictive, and that other MSCs are being missed and could well be found in the remaining GFP population. To further investigate this remaining GFP population gene expression analysis was used on sorted cells (See Chapter 5). An alternate theory is that the GFP population may contain mainly progenitors, MSC derivatives partially committed to particular lineages. With this in mind the osteoblast progenitor was studied in these cells.

This argument can also be switched to ask why the GFP positive percentage of the MSC population is so small? The percentage of the MSC population which are GFP⁺ are as follows: in vivo bone marrow 3.23% and bone 0.99%, in vitro bone marrow normoxic culture 0.82%, hypoxic culture 10.42%, and bone normoxic culture 3.70% and hypoxic culture 10.24%. There are several reasons for such a small proportion of the MSCs being GFP⁺. Firstly, as mentioned before, the three ‘MSC markers’ are too stringent and perhaps this isn’t the true MSC population. Secondly, the MSC population could be a heterogeneous set of cells and so Wt1 is only identifying a subset of these. Thirdly, Wt1 expression may be cyclical and therefore not all cells express Wt1 at any one time. Finally, it may be that Wt1 simply is not a marker for MSCs, but highlights a population of progenitors, and again this was another reason to study progenitor markers.

Wt1 deletion showed little effect on various subpopulations in vivo which is surprising due to the dramatic phenotype (i.e. bone and fat loss, plus reduction in osteoblast synthesis) previously seen in Wt1⁻/⁻ adult mouse organs. However, the
bone and fat loss effect seen in the adult knock out could be due to systemic non-autonomous factors changing, whereas this present study is solely testing for changes in the distribution of MSCs and osteoblast progenitors. There could also be autonomous cell traits, however the cell function has not been identified here, but is the next step of this study. Taking this into account, Wt1 deletion did cause a significant reduction in the MSCs as a proportion of GFP positive bone marrow \((p<0.05)\) and a similar trend was seen in the bone \((p=0.09)\). It is possible that any deletion effect is too subtle to be picked up when cells are freshly analysed and that the rest of the body is compensating in some way for these cells. Therefore the same subpopulations were looked at in vitro. However, in vitro deletion seemed to have an opposite effect, causing an increase in the GFP population in bone \((p<0.05)\) and marrow, but as seen in vivo a slight reduction in the MSCs as a proportion of GFP positive bone \((p=0.41\) and \(p=0.46\) for normoxia and hypoxia respectively), but an increase in hypoxic marrow \((p=0.06)\). It is difficult to know why deletion is having such a small effect in vitro apart from the fact that possibly more time is needed to see any effects, or as previously covered, it is having no effect on distribution, and perhaps adult bone loss is not due to MSCs or intrinsic cell autonomous factors.

The variation seen in GFP expression following Wt1 deletion and also between in vivo and in vitro conditions leads to questions regarding Wt1 regulation. The fact that in vitro Wt1 deletion results in an increase in GFP suggests that some kind of Wt1 regulation is occurring in vivo. Unfortunately, there is little information on any upstream regulators of Wt1. It is known, however, that Wt1 is capable of negative autoregulation in humans (Malik et al., 1994) and mice (Rupprecht et al., 1994). Rupprecht et al. (1994) go on to suggest that the transient nature of Wt1 expression may be due, partially at least, to this negative feedback loop. This could also explain the results seen here. In the Wt1-GFP mice, GFP is knocked in to the ATG of the Wt1 translational start site in the 5’ homologous arm, and therefore will still mirror Wt1 levels as Wt1 negatively regulates itself via its own promoter (Rupprecht et al., 1994; Hosen et al., 2007). One final point for consideration is that the GFP protein has a half life of ~26 hours which may also explain the variation in GFP levels (Corish and Tyler-Smith, 1999).
To determine whether the remaining GFP population was indeed made up of progenitors rather than mesenchymal stem cells, osteoblast progenitor markers were analysed. For bone and marrow *in vivo*, the non-haematopoietic cells were mainly found to be CD166^−^Sca-1^−^ and CD166^+^Sca-1^−^ which are the osteoblast progenitor populations as they contain osteoblasts and are also capable of osteogenesis. The majority of non-haematopoietic GFP^+^ cells were also found in these populations. Perhaps a greater sample size would highlight the subtle effect of *in vivo* *Wt1* deletion as it seems to be causing a trend shift in the differential capabilities of bone and marrow cells. However, compared to *in vivo*, culturing these non-haematopoietic marrow and bone GFP cells *in vitro* alters their expression of CD166 and Sca-1. This is probably because *in vivo* cells are being compared with a cultured, plastic adherent population. There was no effect from deleting *Wt1 in vitro*, however with a sample size of 2 it is unlikely that any subtle changes would be obvious. As with the effect on MSC distribution, the lack of change in osteoblast progenitor distribution only serves to confirm that these cells are not dying. Although, they may have impaired function and ability to differentiate, as was seen by Chau *et al.* (2011), therefore the next step was to assess the differential capabilities of these cells.

Interestingly, *Wt1* deletion is having an effect on the GFP proportion of each quadrant. The percentage of the quadrant which is GFP^+^ is reduced when *Wt1* is deleted. This is the case for bone and marrow, *in vivo* and *in vitro*, showing that although *Wt1* deletion does not affect the distribution of cells it is affecting the number. These preliminary data suggest that *Wt1* is not just a marker for these cells but also regulates their numbers. Also, the quadrant with the highest proportion of GFP^+^ cells is the CD166^+^Sca-1^+^ quadrant, with up to 15% of the quartile being GFP^+^ in hypoxic bone. This population was uncharacterised by Nakamura *et al.* (2010) and so future work would include characterising these cells further.
Chapter 4

Testing the stem/progenitor properties of the Wt1+ cell population in adult bone and bone marrow
4.1. Introduction

In the previous chapter I showed that bone and marrow contain \textit{Wt1} expressing cells and that this population contains osteoblast progenitors and can be related to MSCs. Even though the GFP is such a small percentage of the total and Triple\(^+\) population, these data are still only based on cell surface marker expression. Therefore, the next step was to further investigate the other stem like properties of these cells. As discussed previously, Dominici \textit{et al.} (2006) state that an MSC must have the ability to differentiate into 3 lineages: osteoblasts, chondrocytes, and adipocytes. In addition to this, proliferation without terminal differentiation, another stem cell trait, can be demonstrated using a CFU-F assay as first outlined by Friedenstein \textit{et al.} in 1970, i.e proving that a single cell can result in a colony of cells. Therefore, differentiation capabilities of these cells plus their ability to form colonies from single cells were studied. Of course, a ‘gold standard’ test for a stem cell is to trace its progeny using a lineage tracing technique, and so this was also investigated in adult mice to determine whether \textit{Wt1} positive cells give rise and contribute to particular lineages.

4.2. \textit{In Vitro} differentiation capabilities of GFP positive cells

As before, marrow and bone cells were obtained from \textit{Wt1}-GFP mice, stained, and negatively sorted for lineage markers and the endothelial CD31 marker. The cells were also positively stained for the three MSC cell surface markers (CD29, CD73, and CD105). The Triple\(^+\) cells were sorted into GFP positive and negative populations. These cells were then cultured under the differentiating conditions for the following 3 mesenchymal lineages: adipogenesis, osteogenesis, and chondrogenesis. The use of various markers was highly restrictive on the number of cells which could be obtained. Consequently, the sparse number of cells meant they did not grow well; however, a preliminary study was still carried out. Figure 4.1 shows Lin\(^-\)CD31\(^-\)Triple\(^+\)GFP\(^+\) and GFP\(^-\) populations differentiating into various lineages cultured \textit{in vitro} under hypoxia. The morphology of these cells was compared with the data examples provided by the Mouse Mesenchymal Stem Cell Function Identification Kit Handbook (Cat# SC010, R&D Systems Europe, Ltd., Abingdon, OX14 3NB, UK). Purely based on morphology, it appears that the GFP\(^+\)
cells are able to differentiate into the three lineages (Figure 4.1 C,G,E) and that GFP$^+$
cells are also able to undergo adipogenesis and osteogenesis (Figure 4.1 D,F). Both
Figure 4.1 C and D show cells containing lipid droplets which are characteristic of
fat cells, suggesting that GFP cells can produce adipocytes. However, this is a
preliminary experiment and it would seem that selecting the Triple$^+$ population may,
again, be hindering the characterisation of these cells. Therefore this should be
repeated using Lin$^-$CD31$^-$GFP populations and differentiation verified using the
appropriate staining, such as Oil Red, Alcian Blue, and Alizarin Red. Antibody
staining could also be used to corroborate findings (Adipogenesis: FABP4,
Osteogenesis: Osteopontin, and Chondrogenesis: Collagen II).

4.3. **In Vitro adipogenesis and Wt1**

To ascertain expression levels of *Wt1* during differentiation an adipogenesis time
course was carried out. Total bone marrow cells were cultured under normoxia and
hypoxia for 7 days before adipocyte differentiation media was added for 12 days
once the cells were 80% confluent (usually occurring at 11-12 days of culture). The
percentage of GFP$^+$ cells was analysed using FACS, the percentages of GFP$^+$ cells
observed in the GFP$^-$ controls are false positives due to autofluoresence. Figure 4.2
shows that the percentage of total bone marrow cells which are GFP$^+$ increases
during adipogenesis, peaking at day 5 of differentiation and decreasing by day 12. It
is not known whether the GFP$^+$ proportion increases further between days 5 and 12.
Encouragingly, under the hypoxic conditions, the *Wt1* expression levels follow the
same trend, increasing throughout adipogenesis and peaking again at day 5 (Figure
4.3). This suggests that during adipocyte differentiation, cells start to express GFP
(*Wt1*) increasing the GFP proportion, and that these cells also increase their relative
expression of *Wt1* per cell. Both proportion and expression levels fall by day 12 of
differentiation, by which time the cell has become terminally differentiated. A
greater sample size is required to completely verify these results and a negative
control using non-adipogenic medium needs to be included with an absence of
differentiation media, to ensure that this is not occurring under normal culture
conditions.
Figure 4.1. Preliminary data suggests non-haematopoietic GFP positive MSCs (Lin- CD31-Triple-GFP+) are able to differentiate in vitro into 3 lineages based on morphology. A. Undifferentiated GFP+ marrow cells. B. Undifferentiated GFP- marrow cells. C. Adipogenesis in GFP+ marrow cells. D. Adipogenesis in GFP- marrow cells. E. Osteogenesis in GFP+ bone cells. F. Osteogenesis in GFP- bone cells. G. Chondrogenesis in GFP+ bone cells. H. Chondrogenesis in GFP- bone cells. (Scale bars A,B,D,E,F,G,H = 50 μm, C = 20 μm).
Figure 4.2. The GFP+ percentage of total marrow cells increases during adipogenesis. Total marrow cells from GFP mice (WGER), cultured under normoxia and hypoxia, show an increase in the mean (±SEM) percentage of GFP+ cells from 7 days of culture, to the initial day of differentiation (when 80% confluent, usually day 12 culture), peaking at day 5 of differentiation before reducing by day 12 differentiation. (n=3). GFP negative controls show false positive GFP+ cells due to flow cytometry gating and autofluorescence.

Figure 4.3. Wt1 mRNA expression levels increase during adipogenesis. The mean (±SEM) fold change in Wt1 mRNA expression of bone marrow increases from time point 1 (7 days culture), time point 2 (start of adipocyte differentiation, when cells are 80% confluent, usually at day 12 of culture), time point 3 (5 days differentiation), to time point 4 (12 days differentiation) in normoxia. Under hypoxia, Wt1 levels peak at time point 3 before dropping when terminally differentiated at time point 4. (Time point 1 n=2, time point 2-4 n=6).
4.4. Colony Forming Unit Fibroblast Assays (CFU-F)

CFU-F assays are frequently used to characterise stromal marrow cells (Kuznetsov et al., 1997; Bianco et al., 2001). The adherent stromal cells from murine bone marrow have previously been shown to form colonies originating from a single cell; the colony forming unit-fibroblast (CFU-F) (Friedenstein et al., 1970; Bianco et al., 2001). Marrow and bone from male and female WER mice with a tamoxifen inducible deletion (CAGG-CreERT2;Wt1loxP/loxP) were plated out in a single cell suspension at low and high densities of 0.5 x 10^6 and 1 x 10^6 cells per well respectively (of a 6 well culture plate) (See Section 2.3.5 and Appendix 1). Tamoxifen was added in culture and Figure 4.4 shows a representative image for each well and shows that Wt1 deletion is having a clear effect. The mutant wells (CAGG-CreERT2 positive;Wt1loxP/loxP) show an obvious reduction in colonies. This was verified by counting the colony numbers in the low density wells; total colonies (Figure 4.5) and large colonies (Figure 4.6). Wt1 deletion is having a significant negative effect (p<0.001) on the total number of colonies arising from bone (normoxia and hypoxia, male and female) and marrow (normoxia and hypoxia, but only female). This is the same for the number of large colonies (i.e. colonies of 50 or more cells). Interestingly, the sex of the mice also seems to be having a significant effect (p<0.001), with higher total and large colony numbers in the female marrow (normoxic and hypoxic) but fewer total colonies from the female hypoxic bone.

As well as a reduction in the number of colonies, Wt1 deletion is also causing a reduction in colony size. Figure 4.7 shows that the area and diameter of the total colonies is reduced when Wt1 is knocked out in male mice. The same trend is seen in female mice, however with less significance (Figure 4.8). This could be due to a small sample size (n=2) as it was in the female mice that the greatest effect was seen on the colony numbers. These results therefore suggest that the deletion of Wt1 affects the ability of these cells to form colonies, resulting in fewer and smaller colonies.
Figure 4.4. Representative images for each CFU-F culture well. Cells were cultured under normoxic conditions (A-D) and hypoxic conditions (E-H), and plated out at a low density of $0.5 \times 10^6$ cells per well (A,B,E,F) and a high density of $1 \times 10^6$ cells per well (C,D,G,H). Cells were then stained with 0.5% Cresyl Violet Acetate solution. Colony number and size was compared between control (A,C,E,G) and mutant cells (B,D,F,H). (Scale bars = 5 mm). (n=4).
Figure 4.5. **CFU-F Assay: Total number of colonies is reduced in Wt1 mutant.**

A. **Marrow - Normoxia**

- Mean (±SEM) number of colonies from marrow cultured under normoxia is reduced in female Wt1 mutant (p<0.001) and is increased in females compared to males (p<0.001 control, p<0.05 mutant).

B. **Marrow - Hypoxia**

- Mean (±SEM) number of colonies from marrow cultured under hypoxia is reduced in female Wt1 mutant (p<0.001) and is increased in females compared to males (p<0.001 control, p<0.05 mutant).

C. **Bone - Normoxia**

- Mean (±SEM) number of colonies from bone cultured under normoxia is reduced in both female and male Wt1 mutants (p<0.001).

D. **Bone - Hypoxia**

- Mean (±SEM) number of colonies from bone cultured under hypoxia is reduced in both female and male Wt1 mutants (p<0.001) as well as female controls compared to males (p<0.05). (n=4).
Figure 4.6. CFU-F Assay: Number of large colonies is reduced in Wt1 mutant. A. Mean (±SEM) number of large colonies from marrow cultured under normoxia is reduced in female Wt1 mutant (p<0.001) and is increased in control females compared to males (p<0.001 control). B. Mean (±SEM) number of large colonies from marrow cultured under hypoxia is reduced in female Wt1 mutant (p<0.001) and is increased in females compared to males (p<0.001 control, p<0.01 mutant). C. Mean (±SEM) number of large colonies from bone cultured under normoxia is reduced in both female and male Wt1 mutants (p<0.001). D. Mean (±SEM) number of large colonies from bone cultured under hypoxia is reduced in both female and male Wt1 mutants (p<0.001). (n=4).
Figure 4.7. CFU-F Assay - Male: Area and diameter of colonies are reduced in Wt1 mutant. **A.** Mean (±SEM) area of total colonies from marrow is reduced in Wt1 mutants cultured under hypoxia (p<0.001) and normoxia (p<0.01). **B.** Mean (±SEM) diameter of total colonies from marrow is reduced in Wt1 mutants cultured under hypoxia and normoxia (all p<0.001). **C.** Mean (±SEM) area of large colonies from marrow is reduced in Wt1 mutants cultured under hypoxia and normoxia (all p<0.001), but increased in high density normoxic mutants (p<0.05). **D.** Mean (±SEM) diameter of large colonies from marrow is reduced in Wt1 mutants cultured under low density and hypoxia (p<0.05). (Control n=2, Mutant n=3).
**Figure 4.8. CFU-F Assay - Female: Area and diameter of colonies are reduced in Wt1 mutant.**

A. Mean (±SEM) area of total colonies from marrow is reduced in Wt1 mutants cultured under normoxia at high densities (p<0.001) and low densities (p<0.01). B. Mean (±SEM) diameter of total colonies from marrow is reduced in Wt1 mutants cultured under normoxia (high density p<0.001, low density p<0.01). C. Mean (±SEM) area of large colonies from marrow is reduced in Wt1 mutants cultured at low density under hypoxia (p<0.01). D. Mean (±SEM) diameter of large colonies from marrow is reduced in Wt1 mutants cultured at low density under hypoxia (p<0.05). (n=2).
4.5. Cre toxicity effects

As shown earlier, only a small percentage of the total marrow and bone cells express *Wt1*. It is, therefore, disconcerting that the deletion of *Wt1* in the total population of cells should have such a dramatic effect on the CFU-F assay results and clonogenic capabilities of these cells. Other than their expression of *Wt1*, the only other way these control and mutant mice differ is by the presence or absence of the CreER recombinase. CreER recombinases are developed by fusing Cre to the estrogen receptor, rendering the CreER recombinase inactive (Feil *et al.*, 2009). It can then be activated by 4-OH tamoxifen, which is a synthetic ligand for the estrogen receptor (Feil *et al.*, 2009). Therefore, to test whether CreER recombinase was having any effect I used a mouse line with no *loxp* sites which either contained a tamoxifen inducible CreER recombinase (Cre⁺) or not (Cre⁻). A CFU-F assay was carried out using these cells cultured either with tamoxifen to activate the CreER recombinase or with the carrier EtOH as a control at a low density of 0.5 x 10⁶ cells per well of a 6 well culture plate. Figure 4.9 shows a representation of the CFU-F wells and it is quite clear that activated CreER recombinase is in some way inhibiting the growth of colonies. This is confirmed in Figure 4.10 which shows a significant decrease in the number of colonies when cells are cultured with tamoxifen (i.e. activated CreER recombinase) compared to EtOH (i.e. inactivated CreER recombinase). This is the case in both male and female samples, cultured under normoxia (p<0.01) and hypoxia (p<0.001). This study has also uncovered a fascinating sex effect. In the females only, the Cre⁻ (and therefore control) mice show significantly higher colony numbers when cultured with tamoxifen rather than EtOH (total colonies p<0.001, large colonies p<0.05).

These results suggest that, at least for colony forming capabilities, the cells are affected by the activated CreER recombinase, rather than the loss of *Wt1* deletion. This raises a great concern as to whether activated CreER recombinase is the cause of other phenotypes seen in published studies using this construct.

The data shown in Figure 4.10 also highlights a dramatic difference in colony numbers between ethanol treated Cre⁺ and Cre⁻ cells. All the female cells
(normoxia/hypoxia, total/large) show this increase to be significant \((p<0.001)\) as well as the total number of male colonies cultured under normoxia \((p<0.01)\). As these cells are treated with ethanol the CreER recombinase is not activated suggesting that the mere presence of the CreER construct is the only difference between these cells. In addition, colony numbers were increased in Cre\(^+\) cells which was the opposite effect to that seen when with cultured with tamoxifen.

The aim of this experiment was to assess the effects of CreER recombinase and has so far had uncovered effects of activated Cre, inactivated Cre, and sex. Therefore the final variant to assess was the presence of \(\text{loxP}\) sites. WER mice (i.e. CAGG-CreER\(^{T2}\); \(\text{Wt1}^{\text{loxP/loxP}}\)) used for the control and \(\text{Wt1}\) deletion mutant CFU-F assays (Figure 4.4 - 4.8) differ from the mice used to assess Cre toxicity (i.e. CAGG-CreER\(^{T2}\); \(\text{Wt1}^{+/+}\)) by the presence of \(\text{loxP}\) sites. Table 4.1 compares colony numbers from marrow cells cultured with tamoxifen from CAGG-CreER\(^{T2}\) negative; \(\text{Wt1}^{\text{loxP/loxP}}\) mice (i.e. with \(\text{loxP}\) sites) and CAGG-CreER\(^{T2}\) negative; \(\text{Wt1}^{+/+}\) mice (i.e. without \(\text{loxP}\) sites). The presence of \(\text{loxP}\) sites has no effect on the colony forming abilities of male cells, but does seem to have a positive effect on the number of female colonies. A second difference to consider is possible background effect as WER mice have mixed background compared to the pure background of the CAGG-CreER\(^{T2}\) negative; \(\text{Wt1}^{+/+}\) mice.
Figure 4. A representation of CFU-F wells from 4 mice. Marrow cells from Cre+ mice (1&2) and Cre- mice (3&4) were cultured with Tamoxifen or EtOH, under hypoxic conditions. Cre+ cells cultured with Tamoxifen appear to have fewer colonies. (Scale bar = 5 mm).
Figure 4.10. CFU-F: Activated CreER recombinase results in reduced marrow colony numbers. **A.** Mean (±SEM) total number of male colonies is reduced when Cre is activated by tamoxifen, under normoxia (p<0.01) and hypoxia (p<0.001). **B.** Mean (±SEM) number of large male colonies is reduced when Cre is activated by tamoxifen, under normoxia (p<0.01) and hypoxia (p<0.001). **C.** Mean (±SEM) total number of female colonies is reduced when Cre is activated by tamoxifen, under normoxia (p<0.01) and hypoxia (p<0.001), but is also increased in control subjects cultured with tamoxifen compared to EtOH (p<0.001). **D.** Mean (±SEM) number of large female colonies is reduced when Cre is activated by tamoxifen, under normoxia (p<0.01) and hypoxia (p<0.001), but is also increased in control subjects cultured with tamoxifen compared to EtOH (p<0.05). (n=3).
Table 4.1. Average number (±SEM) of colonies from marrow cells treated with tamoxifen with and without loxP sites

<table>
<thead>
<tr>
<th>Sex</th>
<th>Oxygen conditions</th>
<th>Colonies counted</th>
<th>CAGG-CreER\textsuperscript{T2} negative; Wt1\textsuperscript{loxP/loxP}</th>
<th>CAGG-CreER\textsuperscript{T2} negative; Wt1\textsuperscript{+/+}</th>
<th>T-test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Normoxia</td>
<td>Total</td>
<td>6.92 ± 0.68</td>
<td>7.58 ± 1.54</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td>1.67 ± 0.41</td>
<td>1.75 ± 0.43</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td>Total</td>
<td>11.58 ± 1.03</td>
<td>14.75 ± 3.01</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td>6.17 ± 0.75</td>
<td>6.50 ± 1.56</td>
<td>0.85</td>
</tr>
<tr>
<td>Female</td>
<td>Normoxia</td>
<td>Total</td>
<td>21.67 ± 1.78</td>
<td>20.33 ± 2.09</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td>9.17 ± 1.02</td>
<td>6.11 ± 0.89</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td>Total</td>
<td>42 ± 1.76</td>
<td>20.44 ± 1.96</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td>29.83 ± 1.97</td>
<td>6.67 ± 1.20</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

4.6. Lineage Tracing

To determine whether \textit{Wt1} positive cells give rise and contribute to particular cells and tissues, lineage tracing was carried out. A \textit{Wt1}\textsuperscript{CreERT2};\textit{mTmG} mouse was used in which Tomato is ubiquitously expressed under a pCA promoter, but following Cre mediated \textit{loxP} recombination and the removal of Tomato, GFP is expressed. This allows cells with activated CreER recombinase (driven by the \textit{Wt1} promoter) to be “tattooed” at the time of tamoxifen induction. These GFP-expressing “tattooed” cells can then be traced as well as their progeny. Figure 4.11 is a schematic cartoon to illustrate the mechanism involved in lineage tracing.

![Figure 4.11. Lineage Tracing. The cell expresses Tomato (red), tamoxifen is added and CreER recombinase activated resulting in \textit{loxP} site recombination. Tomato is excised and GFP is now expressed making the cell, and its progeny, permanently green from that moment onward.]
4.6.1. *In Vivo* Lineage Tracing

Lineage tracing was carried out in adult mice at varying dosages and for varying lengths of time. Table 4.2 shows the number of doses administered and the length of time between dosing and harvesting. It also shows that no GFP was seen in either bone or marrow, and very little or no GFP seen in the positive control spleen (Representative flow cytometry plots can be found in Appendix 6). Evidently there is a problem with our system as it has already been demonstrated that bone and marrow contain *Wt1* expressing cells (See Chapter 3, specifically Figure 3.1, and Hosen *et al.*, 2002). Theoretically the results from the lineage tracing should show GFP cells of at least the same number if not more than the endogenous *Wt1* expressing cells. Possible explanations for this are not enough tamoxifen is being administered, which is why the dosages where increased from 2 to 4. 4 doses should be adequate therefore it is not likely for this to be the cause. Another aspect could be that administration by gavaging is not effectively delivering the tamoxifen to the bone and marrow. However, gavaging has previously been shown to deliver tamoxifen to the spleen effectively, so the absence of high GFP cell numbers in the spleen also suggests that this is probably not the problem. Although it does not explain the lack of GFP expression, it is possible that the adult age of these mice may be playing a role. As the majority of bone development occurs by late development (~e16.5) I felt it would be worth repeating this with embryonic and early postnatal mice (See Chapter 6).

### Table 4.2. *In vivo* lineage tracing

<table>
<thead>
<tr>
<th>Number of Doses</th>
<th>Time between dosing and harvesting</th>
<th>Sample Size</th>
<th>Age (weeks)</th>
<th>GFP seen in bone</th>
<th>GFP seen in marrow</th>
<th>GFP seen in spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4 weeks</td>
<td>3 Females</td>
<td>8</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>2 Days</td>
<td>1 Male</td>
<td>7</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>1 Day</td>
<td>2 Males</td>
<td>7</td>
<td>None</td>
<td>None</td>
<td>Very Little</td>
</tr>
<tr>
<td>4</td>
<td>3 Days</td>
<td>3 Males</td>
<td>16</td>
<td>None</td>
<td>None</td>
<td>Very Little</td>
</tr>
<tr>
<td>4</td>
<td>3 Weeks</td>
<td>3 Males</td>
<td>16</td>
<td>None</td>
<td>None</td>
<td>Very Little</td>
</tr>
<tr>
<td>4</td>
<td>6 Weeks</td>
<td>3 Males</td>
<td>8</td>
<td>None</td>
<td>None</td>
<td>Very Little</td>
</tr>
</tbody>
</table>
4.7. Discussion

The results from this chapter have confirmed that cells from the bone and marrow, cultured under MSC favouring conditions, are capable of forming colonies from a single cell. Proliferation without differentiation is a stem cell trait and thus stem cells must be present in this population. To repeat the CFU-F assay using a purely GFP positive population would be beneficial to this study. It was attempted during this project, however once sorted the GFP\(^+\) cells grow very poorly. It is unknown as to whether this is due to the sorting procedure or the lack of paracrine communication between the GFP\(^+\) and GFP\(^-\) populations. To add to the evidence of a stem-like population within the bone and marrow cells, it appears that the non-haematopoietic GFP sorted cells are capable of differentiating into the three main lineages. Of course, this still needs verifying with appropriate cell staining of markers for each lineage. These preliminary data, however, are encouraging. To add to this, the associated rise in \(Wt1\) expression and GFP positive cells during the adipogenesis process suggests the gene may be playing a role in the adipocyte progenitor population, but less so in the terminally differentiated fat cells. This is interesting as recent work has shown \(Wt1\)-GFP positive cells to be present in the fat progenitor population, but absent from the mature fat cell population (Chau et al., 2014).

Although progress is being made in characterising the endogenous \(Wt1\) expressing GFP cells, several technical difficulties have been encountered whilst investigating the functional role of \(Wt1\). Figures 4.9 and 4.10 confirm that the dramatic effect of \(Wt1\) deletion on the ability of these cells to form colonies is actually due to the activated CreER recombinase, rather than loss of gene expression. This is a worrying finding, not just for this project, but for the scientific community as a whole. Very few studies control for CreER toxicity effects and may be wrongly accrediting their findings to a changed gene expression status. The initial ubiquitous deletion of \(Wt1\) by Chau et al. (2011) which sparked the interest in this topic was controlled for using Cre\(^+\) mice with no \(loxP\) sites. There were no significant effects on tissue morphology as observed by pathologists following CreER activation \textit{in vivo}, however CFU-F assays were not checked (Chau et al., 2011). The lack of
morphological effects is possibly because the body is better at coping with Cre toxicity by removing it from the cells or compensating for its effects, unlike in vitro cells which are cultured in wells and unable to escape the activated CreER recombinase. It is interesting to speculate why activating CreER recombinase is creating this phenotype in vitro and there are 2 potential explanations: Firstly, the CreER recombinase protein is toxic. Secondly, the CreER recombinase is acting on endogenous pseudo-loxP sites and is causing off target effects. The data from this chapter showed that inactivated CreER recombinase is also having an effect when compared to Cre negative samples. However, this effect is positive on colony forming abilities, opposite to the negative effects of activated CreER recombinase.

The CreER control CFU-F results also uncovered an interesting sex effect on the wild type mice. Cells from the female mice resulted in much higher colony numbers when cultured with tamoxifen, as opposed to EtOH. There are links between tamoxifen, the estrogen receptor, and bone growth which make this an interesting observation that will be reviewed in the discussion chapter of this thesis. However, tamoxifen may not be entirely responsible for this increase in female colony numbers as it would seem that the presence of loxP sites is also having a sex-specific positive effect on female colony forming abilities.

Another technical hitch occurred with the use of the Wt1\textsuperscript{CreERT2};mTmG lineage tracing mice. As already considered, the lack of GFP cells observed in vivo could be due to a number of factors: dosage, administration, timing, age, sample preparation methods, or another alternative fundamental reason. Therefore, lineage tracing was readdressed during this project using younger mice (See Chapter 6).

A constitutively active Wt1-Cre model linked with YFP (Wt1-Cre;YFP) has also been used as a lineage tracing model in the bone marrow by Ramón Muños-Chápuli. However, 2 of the 3 times this has been carried out no YFP signal was detected in the marrow cells (Personal communication between the Hastie laboratory and Ramón Muños-Chápuli). It seems that Wt1 lineage tracing in the adult bone and marrow is unpredictable despite it working beautifully in other tissues. It is possible that this is the result of transgene silencing in these particular cell types and it would be interesting to study the methylation statuses of these cells.
Chapter 5

Pathways affected by $Wt1$ deletion in bone and bone marrow
5.1. Introduction

The data thus far have shown the GFP+ population to contain possible MSCs, osteoblast progenitors, and potentially be capable of tri-lineage differentiation. The loss of Wt1 expression has had little effect on either MSC or osteoblast subpopulation distributions. Therefore, gene expression microarrays were carried out to determine what role Wt1 was playing at a transcriptional level. The purpose of this was to identify what genes and pathways are active in the bone and marrow and what functional role Wt1 plays with respect to these genes and pathways.

Two sets of microarrays were carried out: 1) cultured total bone and marrow cells from CAGG-CreER\textsuperscript{TM},Wt1\textsuperscript{loxP/loxP} (WER) mice with the tamoxifen inducible Wt1 deletion, under normoxia and hypoxia, and 2) non-haematopoietic (Lin-CD31-) bone and marrow cells, also with the tamoxifen inducible Wt1 deletion, from CAGG-CreER\textsuperscript{TM},Wt1\textsuperscript{loxP/GFP} (WGER) mice sorted positively and negatively for GFP expression (See Section 2.8 and Appendix 4 & 5). Comparisons were made between control and mutant samples, normoxic and hypoxic, as well as GFP+ and GFP- in the second set of arrays. As shown in the previous chapter, CreER recombinase was having a negative effect on the ability of marrow and bone cells to form colonies. Therefore, a third microarray was run comparing hypoxic bone cultured from the CAGG-CreER\textsuperscript{TM} mice which lack the loxP sites. Cre- bone cells were compared against Cre+ (i.e. activated CreER recombinase) cells. The result from this comparison was then used to compensate the other microarray sets and determine whether activated CreER was having an effect on gene expression levels, or whether the changes were indeed due to loss of Wt1 expression.

5.2. Total cell microarrays

The functional role of Wt1 was assessed by comparing differentially expressed genes in control cells compared to mutant (i.e. Wt1 deletion) cells. This comparison was carried out in both marrow and bone, cultured under normoxia and hypoxia. Control and mutant marrow cells differed significantly (q<0.05) in the expression of 45 genes (Figure 5.1), whereas 8,714 genes were altered by Wt1 deletion in bone cells (Figure
5.2). Using microarray expression analysis of these transcriptional changes I investigated genes and pathways which could explain the bone and fat loss phenotype seen in the *in vivo* WtI adult deletion (Chau *et al.*, 2011).

*Figure 5.1. The breakdown of 45 differential gene expression changes between control marrow cells and mutant marrow cells (n=3).*

*Figure 5.2. The breakdown of 8,714 differential gene expression changes between control bone cells and mutant bone cells (n=3).*
Analysis was carried out on these genes using two gene ontology tools: GOrilla (http://cbl-gorilla.cs.technion.ac.il/) and DAVID (http://david.abcc.ncifcrf.gov/). The gene ontology enrichment analysis and visualization tool (GOrilla) identifies gene ontology (GO) terms from a ranked list of genes, e.g. by log fold change in expression. The Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 allows users to use functional annotation tools to highlight pathway maps as well as GO terms. The 45 changes in gene expression seen in the marrow is a relatively small gene number compared to the bone’s 8,714 genes. The four marrow subsets (i.e. gene expression increasing or decreasing, under normoxia or hypoxia) are too small to result in GO Term enrichment. However, several of the genes are linked with mesenchymal lineages, including fat, muscle, bone, and cartilage. These are outlined in Tables 5.1-5.3.

The 8,714 genes that changed significantly (q<0.05 calculated by the R bioconductor package LIMMA) under hypoxia in the bone were ordered by log fold change and analysed in GOrilla (last updated November 9th 2013). Enrichment values were calculated by the GOrilla tool which takes into account the total number of genes from the microarray, the total number of genes associated with a specific GO term, and the number and ranking of those associated genes which are present in the microarray output.

5.2.1. GO terms down regulated in Wt1 deletion mutant bone cells

Genes which were down regulated in the Wt1 deletion mutant bone cells were enriched for 109 GO terms with an FDR (False Discovery Rate) q-value <0.05 (adjusted p-value using the Benjamini and Hochberg (1995) method). These GO terms were associated with several areas, but largely developmental processes. Table 5.4 shows enriched GO terms which are of particular interest to this study. These include the development of mesoderm, cartilage, tooth mineralization, blood vessels, and skeletal systems, all of which originate from mesenchymal cells. Figure 5.3 is the corresponding visual flow chart taken from GOrilla showing the connection and significance of each GO term. The 109 down regulated GO terms also included various muscle tissue morphogenesis events, muscle contraction, and the collagen biosynthetic process as shown in Table 5.5 and Figure 5.4. Again, these are events
associated with skeletal bone formation and mesenchymal lineage. Table 5.6 shows a selection of other GO terms either of high enrichment value or of relevance and interest to this project. DAVID was used to confirm the enriched GO terms down regulated in the \textit{Wt1} mutants by using microarray gene changes with a p value \textless{}0.05 and a log fold change \textgreater{}1.5. Table 5.7 shows a selection of GO terms from DAVID which were also identified by GOrilla, including bone development.

\subsection*{5.2.2. GO terms up regulated in \textit{Wt1} deletion mutant bone cells}

Genes which were up regulated in the \textit{Wt1} deletion mutant bone cells, however, were mainly enriched for immune response GO terms. This is shown by the GO terms in Table 5.8, and the corresponding flow charts taken from the GOrilla visualization tool (Figures 5.5). Again, this was confirmed by DAVID using microarray gene changes with a p value \textless{}0.05 and a log fold change \textgreater{}1.5. Results showed enrichment in similar immune response GO terms at comparable q-values (Table 5.9).

\subsection*{5.2.3. GO terms altered by oxygen level}

As these total cell microarrays were carried out on normoxic and hypoxic cells this allowed a comparison to be made between the two.

The first data set was looking at the genes up regulated in the bone cells cultured in hypoxia, compared to normoxia. There were only 7 enriched GO terms identified by GOrilla with a q value \textless{}0.05 and when cross checked in DAVID (gene changes with p\textless{}0.05 and log fold change \textgreater{}1.5) no GO terms were found to be enriched. The 7 terms identified by GOrilla are shown in Table 5.10. Interestingly the top term is Glycolysis, and unsurprisingly the following 3 terms are responses to oxygen levels (GOrilla visualization shown in Figure 5.6).

Next I looked at the GO terms enriched in genes which were down regulated in hypoxia. These were mainly immune responses, but also included bone remodeling
and resorption, and lipid biosynthesis terms (Table 5.11 and Figure 5.7). Again, DAVID confirmed the immune response GO terms (Table 5.12).

**Table 5.1. Genes increased in Wt1 deletion mutant marrow cells cultured under normoxia**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log Fold Change</th>
<th>Brief Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serpinf1</td>
<td>1.63</td>
<td>Neurotrophic inhibitor of angiogenesis</td>
</tr>
<tr>
<td>Bmp1</td>
<td>1.54</td>
<td>Induces cartilage and bone formation in vivo</td>
</tr>
<tr>
<td>Acta2</td>
<td>1.44</td>
<td>Contractile alpha actin found in skeletal muscle</td>
</tr>
<tr>
<td>Igfbp7</td>
<td>1.36</td>
<td>Binds IGF-I and IGF-II</td>
</tr>
<tr>
<td>Palld</td>
<td>1.29</td>
<td>Organizes actin cytoskeleton</td>
</tr>
<tr>
<td>Pla1a</td>
<td>0.45</td>
<td>Phospholipase which hydrolyzes fatty acids</td>
</tr>
</tbody>
</table>

**Table 5.2. Genes increased in Wt1 deletion mutant marrow cells cultured under hypoxia**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log Fold Change</th>
<th>Brief Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fbxo38</td>
<td>0.37</td>
<td>Coactivates KLF7 which deregulates adipocytokine secretion in adipocytes</td>
</tr>
</tbody>
</table>

**Table 5.3. Genes decreased in Wt1 deletion mutant marrow cells cultured under hypoxia**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log Fold Change</th>
<th>Brief Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atf4</td>
<td>-1.86</td>
<td>Regulates glucose homeostasis with FOXO1 in osteoblasts</td>
</tr>
</tbody>
</table>

**Table 5.4. Enriched GOrilla GO terms of genes down regulated in Wt1 deletion mutant bone**

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0048856</td>
<td>Anatomical Structure Development</td>
<td>188</td>
<td>1.58</td>
<td>1.08 x 10^-7</td>
</tr>
<tr>
<td>GO:0048513</td>
<td>Organ development</td>
<td>88</td>
<td>1.71</td>
<td>5.38 x 10^-4</td>
</tr>
<tr>
<td>GO:0001501</td>
<td>Skeletal system development</td>
<td>22</td>
<td>2.69</td>
<td>8.52 x 10^-2</td>
</tr>
<tr>
<td>GO:0001568</td>
<td>Blood vessel development</td>
<td>19</td>
<td>3.12</td>
<td>5.22 x 10^-2</td>
</tr>
<tr>
<td>GO:0007498</td>
<td>Mesoderm development</td>
<td>8</td>
<td>4.38</td>
<td>4.02 x 10^-2</td>
</tr>
<tr>
<td>GO:0051216</td>
<td>Cartilage development</td>
<td>13</td>
<td>3.61</td>
<td>2.05 x 10^-2</td>
</tr>
<tr>
<td>GO:0034505</td>
<td>Tooth mineralization</td>
<td>2</td>
<td>110.54</td>
<td>2.02 x 10^-2</td>
</tr>
</tbody>
</table>
Figure 5.3. GO terms branching from anatomical structure development shown by GOrilla visualization tool.

Table 5.5. Enriched GOrilla GO terms of genes down regulated in Wt1 deletion mutant bone

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0032502</td>
<td>Developmental process</td>
<td>292</td>
<td>1.45</td>
<td>6.15 x 10^{-9}</td>
</tr>
<tr>
<td>GO:0009887</td>
<td>Organ morphogenesis</td>
<td>41</td>
<td>1.92</td>
<td>2.87 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0003208</td>
<td>Cardiac ventricle morphogenesis</td>
<td>4</td>
<td>12.32</td>
<td>3.99 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0055010</td>
<td>Ventricular cardiac muscle tissue morphogenesis</td>
<td>10</td>
<td>3.87</td>
<td>2.2 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0003012</td>
<td>Muscle system process</td>
<td>14</td>
<td>7.27</td>
<td>3.63 x 10^{-3}</td>
</tr>
<tr>
<td>GO:0060048</td>
<td>Cardiac muscle contraction</td>
<td>5</td>
<td>13.16</td>
<td>2.01 x 10^{-5}</td>
</tr>
<tr>
<td>GO:0016477</td>
<td>Cell migration</td>
<td>52</td>
<td>1.92</td>
<td>2.94 x 10^{-3}</td>
</tr>
<tr>
<td>GO:0032864</td>
<td>Collagen biosynthetic process</td>
<td>5</td>
<td>11.46</td>
<td>8.72 x 10^{-3}</td>
</tr>
</tbody>
</table>
Figure 5.4. GO terms branching from developmental process shown by GOrilla visualization tool.

Table 5.6. Enriched GOrilla GO terms of genes down regulated in Wt1 deletion mutant bone cells

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0019547</td>
<td>Arginine catabolic process to ornithine</td>
<td>1</td>
<td>5748.00</td>
<td>2.07 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0090263</td>
<td>Positive regulation of canonical Wnt receptor signalling pathway</td>
<td>10</td>
<td>5.04</td>
<td>7.72 x 10^{-3}</td>
</tr>
<tr>
<td>GO:0048010</td>
<td>Vascular endothelial growth factor receptor signaling pathway</td>
<td>9</td>
<td>4.65</td>
<td>2.04 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0045765</td>
<td>Regulation of angiogenesis</td>
<td>15</td>
<td>3.25</td>
<td>3.63 x 10^{-4}</td>
</tr>
<tr>
<td>GO:0001666</td>
<td>Response to hypoxia</td>
<td>14</td>
<td>4.60</td>
<td>3.22 x 10^{-3}</td>
</tr>
</tbody>
</table>
Table 5.7. Enriched DAVID GO terms of genes downregulated in Wt1 deletion mutant bone

<table>
<thead>
<tr>
<th>Description</th>
<th>Number of Genes</th>
<th>Percentage (Involved genes / Total Genes)</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal system development</td>
<td>29</td>
<td>5.1</td>
<td>3.7 x 10^-6</td>
</tr>
<tr>
<td>Vasculature development</td>
<td>22</td>
<td>3.8</td>
<td>6.2 x 10^-4</td>
</tr>
<tr>
<td>Collagen fibril organization</td>
<td>7</td>
<td>1.2</td>
<td>6.2 x 10^-4</td>
</tr>
<tr>
<td>Bone development</td>
<td>13</td>
<td>2.3</td>
<td>3.5 x 10^-4</td>
</tr>
</tbody>
</table>

Table 5.8. Enriched GOrilla GO terms of genes upregulated in Wt1 deletion mutant bone

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0002376</td>
<td>Immune system process</td>
<td>106</td>
<td>3.38</td>
<td>4.57 x 10^-26</td>
</tr>
<tr>
<td>GO:0019886</td>
<td>Antigen processing and presentation of exogenous peptide antigen via MHC class II</td>
<td>10</td>
<td>12.10</td>
<td>1.73 x 10^-7</td>
</tr>
<tr>
<td>GO:0002682</td>
<td>Regulation of immune system process</td>
<td>80</td>
<td>3.16</td>
<td>2.66 x 10^-16</td>
</tr>
<tr>
<td>GO:0050778</td>
<td>Positive regulation of immune response</td>
<td>33</td>
<td>4.62</td>
<td>4.54 x 10^-10</td>
</tr>
<tr>
<td>GO:0002819</td>
<td>Regulation of adaptive immune response</td>
<td>19</td>
<td>4.49</td>
<td>1.79 x 10^-5</td>
</tr>
</tbody>
</table>
Figure 5.5. Immune response GO terms shown by GOrilla visualization tool.
Table 5.9. Enriched DAVID GO terms of genes up regulated in Wt1 deletion mutant bone

<table>
<thead>
<tr>
<th>Description</th>
<th>Number of Genes</th>
<th>Percentage (Involved genes / Total Genes)</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response</td>
<td>60</td>
<td>20.6</td>
<td>5.4 x 10^-33</td>
</tr>
<tr>
<td>Defense response</td>
<td>40</td>
<td>13.7</td>
<td>3.3 x 10^-15</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>29</td>
<td>10.0</td>
<td>1.8 x 10^-14</td>
</tr>
<tr>
<td>Immune effector process</td>
<td>22</td>
<td>7.6</td>
<td>3.9 x 10^-13</td>
</tr>
<tr>
<td>Positive regulation of immune system process</td>
<td>26</td>
<td>8.9</td>
<td>1.1 x 10^-12</td>
</tr>
</tbody>
</table>

Table 5.10. Enriched GOrilla GO terms of genes up regulated in bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006096</td>
<td>Glycolysis</td>
<td>9</td>
<td>6.22</td>
<td>2.65 x 10^-2</td>
</tr>
<tr>
<td>GO:0001666</td>
<td>Response to hypoxia</td>
<td>14</td>
<td>4.24</td>
<td>3.57 x 10^-2</td>
</tr>
<tr>
<td>GO:0036293</td>
<td>Response to decreased oxygen levels</td>
<td>14</td>
<td>4.24</td>
<td>2.38 x 10^-2</td>
</tr>
<tr>
<td>GO:0070482</td>
<td>Response to oxygen levels</td>
<td>14</td>
<td>4.24</td>
<td>1.79 x 10^-2</td>
</tr>
<tr>
<td>GO:0044767</td>
<td>Single-organism developmental process</td>
<td>187</td>
<td>1.32</td>
<td>3.36 x 10^-2</td>
</tr>
<tr>
<td>GO:0044699</td>
<td>Single-organism process</td>
<td>121</td>
<td>1.33</td>
<td>4.79 x 10^-2</td>
</tr>
<tr>
<td>GO:0042127</td>
<td>Regulation of cell proliferation</td>
<td>68</td>
<td>1.68</td>
<td>4.38 x 10^-2</td>
</tr>
</tbody>
</table>

Figure 5.6. Hypoxic response GO terms shown by GOrilla visualization tool.
Table 5.11. Enriched GOrilla GO terms of genes down regulated in bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0002376</td>
<td>Immune system process</td>
<td>53</td>
<td>3.88</td>
<td>1.1 x 10^{-13}</td>
</tr>
<tr>
<td>GO:0006952</td>
<td>Defense response</td>
<td>31</td>
<td>3.77</td>
<td>6.91 x 10^{-7}</td>
</tr>
<tr>
<td>GO:0050776</td>
<td>Regulation of immune response</td>
<td>25</td>
<td>4.46</td>
<td>8.18 x 10^{-7}</td>
</tr>
<tr>
<td>GO:0002675</td>
<td>Positive regulation of acute inflammatory response</td>
<td>5</td>
<td>8.25</td>
<td>1.44 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0050715</td>
<td>Positive regulation of cytokine secretion</td>
<td>7</td>
<td>7.04</td>
<td>9.14 x 10^{-3}</td>
</tr>
<tr>
<td>GO:0046890</td>
<td>Regulation of lipid biosynthetic process</td>
<td>5</td>
<td>11.58</td>
<td>2.01 x 10^{-4}</td>
</tr>
<tr>
<td>GO:0034374</td>
<td>Low-density lipoprotein particle remodeling</td>
<td>2</td>
<td>418.12</td>
<td>1.40 x 10^{-3}</td>
</tr>
<tr>
<td>GO:0046850</td>
<td>Regulation of bone remodeling</td>
<td>6</td>
<td>7.13</td>
<td>2.04 x 10^{-4}</td>
</tr>
<tr>
<td>GO:0045780</td>
<td>Positive regulation of bone resorption</td>
<td>3</td>
<td>14.80</td>
<td>2.45 x 10^{-4}</td>
</tr>
</tbody>
</table>

Figure 5.7. Bone remodelling GO terms shown by GOrilla visualization tool.

Table 5.12. Enriched DAVID GO terms of genes down regulated in bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>Description</th>
<th>Number of Genes</th>
<th>Percentage (Involved genes / Total Genes)</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response</td>
<td>21</td>
<td>11.5</td>
<td>1.1 x 10^{-5}</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>14</td>
<td>7.7</td>
<td>3.9 x 10^{-5}</td>
</tr>
<tr>
<td>Defense Response</td>
<td>19</td>
<td>10.4</td>
<td>3.9 x 10^{-5}</td>
</tr>
<tr>
<td>Positive regulation of response to stimulus</td>
<td>12</td>
<td>6.6</td>
<td>1.2 x 10^{-4}</td>
</tr>
<tr>
<td>Positive regulation of immune response</td>
<td>10</td>
<td>5.5</td>
<td>3.0 x 10^{-4}</td>
</tr>
</tbody>
</table>
5.3. Cre control microarrays

Activation of CreER recombinase had a dramatic effect on the ability of bone and marrow cells to form colonies. To assess for the toxicity of activated CreER recombinase and to investigate involved gene pathways bone was cultured under hypoxia from the CAGG-CreERTM;Wt1+/− mice which lack loxP sites. Tamoxifen was added to activate CreER recombinase in the Cre+ cells, and to the Cre− cells as a control.

Encouragingly there were only 15 genes which differentially changed with a q-value less than 0.05. None of these 15 genes had a log fold change greater than 1.5, which were the consistent cut-off values I used on the previous microarray analysis. 7 genes were down regulated and the remaining 8 were upregulated in the Cre+ bone cells. These genes are shown in Table 5.13.

The GOrilla tool uses the entire set of differentially expressed genes ordered by log fold change, irrespective of their q value. The GO terms which were most significantly down regulated in the Cre+ bone cells (i.e. bone cultured under hypoxia with activated CreER recombinase) were associated with RNA processing events as shown in Table 5.14 and Figure 5.8.

On the other hand, various GO terms were enriched for in the genes that were up regulated when CreER recombinase was activated (i.e. in Cre+ bone cells). These were mainly immune response related terms (Table 5.15). However, worryingly there was an enrichment of 4 GO terms relating to bone homeostasis (Table 5.16 and Figure 5.9). Bone resorption, regulation of bone resorption, regulation of bone remodelling, and regulation of bone mineralization are GO terms which consist of 20 genes that were differentially upregulated in Cre+ bone cells, compared to Cre−. Although the 4 bone GO terms were deemed significant (i.e. q values <0.05), none of the 20 genes involved were upregulated with a q value <0.05 and only 2 genes had a log fold change greater than 1.5 (Table 5.17).

Bone resorption and increased osteoclasts were seen when Wt1 was deleted in vivo. However, no comparable phenotype was observed in the in vivo CAGG-CreERTM positive;Wt1+/+ mice (Chau et al., 2011). The CAGG-CreERTM Cre+ and Cre−
control data was normalised by Graeme Grimes from the HGU IGMM against the WER bone cells (CAGG-CreER™;Wt1loxP/loxP Cre- control and Cre+ Wt1 mutant) from the total cell microarray. This is shown in Figure 5.10. Gene expression was normalised to compensate for variations such as batch effects. Principal component analysis and a cluster dendrogram were used to show that when normalised together there is an obvious difference in expression between the WER CAGG-CreER™ positive;Wt1loxP/loxP mutant cells and the rest of the normalised data, i.e. WER CAGG-CreER™ negative;Wt1loxP/loxP control cells, CAGG-CreER™;Wt1+/+ Cre+, and Cre- cells (Figure 5.11). This alone suggests that CreER activation was not having a significant effect. To add to this, when the differentially expressed genes were ordered in the GOrilla tool by their statistical significance (i.e. q-value), rather than log fold change, there was no enrichment for GO terms associated with bone resorption or remodelling. Combined with the fact that none of the 20 associated genes were differentially expressed above a statistical threshold (i.e. a q value of 0.05) and these cells were from an in vitro model and therefore less comparable to the in vivo results, I am inclined to see these GO terms as false positives in the data set. However, future work will include carrying out qRT-PCRs on these genes for complete verification.

**Table 5.13. Genes which are significantly differentially expressed in CAGG-CreER™ positive;Wt1+/+ (Cre-) bone cells**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Log Fold Change</th>
<th>q value (adjusted p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccng1</td>
<td>1.484393</td>
<td>0.013971</td>
</tr>
<tr>
<td>Tgfb1/11</td>
<td>-1.13712</td>
<td>0.02143</td>
</tr>
<tr>
<td>Snx16</td>
<td>1.261051</td>
<td>0.02143</td>
</tr>
<tr>
<td>Cdc20</td>
<td>-1.08877</td>
<td>0.037901</td>
</tr>
<tr>
<td>LOC433464</td>
<td>1.423034</td>
<td>0.037901</td>
</tr>
<tr>
<td>Anxa11</td>
<td>1.49537</td>
<td>0.037901</td>
</tr>
<tr>
<td>Sdc1</td>
<td>0.744466</td>
<td>0.041589</td>
</tr>
<tr>
<td>Rps24</td>
<td>-1.01406</td>
<td>0.047998</td>
</tr>
<tr>
<td>Gins1</td>
<td>-0.94639</td>
<td>0.047998</td>
</tr>
<tr>
<td>Vars</td>
<td>-0.8713</td>
<td>0.047998</td>
</tr>
<tr>
<td>Dyrk1b</td>
<td>-0.78882</td>
<td>0.047998</td>
</tr>
<tr>
<td>Dak</td>
<td>1.050523</td>
<td>0.047998</td>
</tr>
<tr>
<td>LOC100046211</td>
<td>-0.63169</td>
<td>0.049673</td>
</tr>
<tr>
<td>Csf2rb</td>
<td>0.984073</td>
<td>0.049673</td>
</tr>
<tr>
<td>Gna12</td>
<td>1.433287</td>
<td>0.049673</td>
</tr>
</tbody>
</table>
Table 5.14. Enriched GOrilla GO terms of genes down regulated in Cre⁺ bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0008380</td>
<td>RNA splicing</td>
<td>49</td>
<td>2.75</td>
<td>2.74 x 10⁻⁶</td>
</tr>
<tr>
<td>GO:0006396</td>
<td>RNA processing</td>
<td>81</td>
<td>2.05</td>
<td>1.27 x 10⁻⁵</td>
</tr>
<tr>
<td>GO:0016071</td>
<td>mRNA metabolic process</td>
<td>61</td>
<td>2.26</td>
<td>2.46 x 10⁻⁵</td>
</tr>
<tr>
<td>GO:0006397</td>
<td>mRNA processing</td>
<td>49</td>
<td>2.55</td>
<td>1.98 x 10⁻⁵</td>
</tr>
</tbody>
</table>

Figure 5.8. RNA related GO terms shown by the GOrilla visualization tool to be down regulated in Cre⁺ bone cells.
Table 5.15. Enriched GOrilla GO terms of genes up regulated in Cre+ bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0048583</td>
<td>Regulation of response to stimulus</td>
<td>235</td>
<td>1.70</td>
<td>3.95 x 10^-11</td>
</tr>
<tr>
<td>GO:0002682</td>
<td>Regulation of immune system process</td>
<td>99</td>
<td>2.30</td>
<td>9.1 x 10^-11</td>
</tr>
<tr>
<td>GO:0002376</td>
<td>Immune system process</td>
<td>112</td>
<td>2.18</td>
<td>8.24 x 10^-10</td>
</tr>
<tr>
<td>GO:0002495</td>
<td>Antigen processing and presentation of peptide antigen via MHC class II</td>
<td>11</td>
<td>9.58</td>
<td>4.42 x 10^-7</td>
</tr>
<tr>
<td>GO:0006955</td>
<td>Immune response</td>
<td>13</td>
<td>10.27</td>
<td>7.3 x 10^-7</td>
</tr>
<tr>
<td>GO:0006952</td>
<td>Defense response</td>
<td>15</td>
<td>5.47</td>
<td>1.12 x 10^-4</td>
</tr>
</tbody>
</table>

Table 5.16. Enriched GOrilla GO terms of genes up regulated in Cre+ bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0045124</td>
<td>Regulation of bone resorption</td>
<td>3</td>
<td>38.41</td>
<td>1.46 x 10^-2</td>
</tr>
<tr>
<td>GO:0030500</td>
<td>Regulation of bone mineralization</td>
<td>11</td>
<td>4.00</td>
<td>1.84 x 10^-2</td>
</tr>
<tr>
<td>GO:0045453</td>
<td>Bone resorption</td>
<td>8</td>
<td>4.70</td>
<td>2.06 x 10^-2</td>
</tr>
<tr>
<td>GO:0046850</td>
<td>Regulation of bone remodelling</td>
<td>3</td>
<td>30.41</td>
<td>2.45 x 10^-2</td>
</tr>
</tbody>
</table>

Figure 5.9. Bone homeostasis related GO terms shown by the GOrilla visualization tool to be up regulated in Cre+ bone cells
Table 5.17. Genes associated with the bone homeostasis GO terms enriched in the CAGG-CreER positive; Wt1	extsuperscript{+/−} (Cre	extsuperscript{−}) bone cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Name</th>
<th>Associated GO term</th>
<th>Log Fold Change</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csf1r</td>
<td>colony stimulating factor 1 receptor</td>
<td>Regulation of bone remodelling and bone resorption</td>
<td>1.24</td>
<td>0.23</td>
</tr>
<tr>
<td>Trf</td>
<td>transferrin</td>
<td>Regulation of bone remodelling and bone resorption</td>
<td>1.50</td>
<td>0.08</td>
</tr>
<tr>
<td>Tnfrsf11b</td>
<td>tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)</td>
<td>Regulation of bone remodelling and bone resorption</td>
<td>1.36</td>
<td>0.22</td>
</tr>
<tr>
<td>Rab7</td>
<td>rab7, member ras oncogene family</td>
<td>Bone resorption</td>
<td>0.44</td>
<td>0.10</td>
</tr>
<tr>
<td>Rac2</td>
<td>ras-related c3 botulinum substrate 2</td>
<td>Bone resorption</td>
<td>0.93</td>
<td>0.14</td>
</tr>
<tr>
<td>Pthr1</td>
<td>parathyroid hormone 1 receptor</td>
<td>Bone resorption</td>
<td>0.52</td>
<td>0.09</td>
</tr>
<tr>
<td>Ptk2b</td>
<td>ptk2 protein tyrosine kinase 2 beta</td>
<td>Bone resorption</td>
<td>0.69</td>
<td>0.14</td>
</tr>
<tr>
<td>Src</td>
<td>rous sarcoma oncogene</td>
<td>Bone resorption</td>
<td>0.57</td>
<td>0.27</td>
</tr>
<tr>
<td>Ctsb</td>
<td>cathepsin s</td>
<td>Bone resorption</td>
<td>0.72</td>
<td>0.47</td>
</tr>
<tr>
<td>Xdh</td>
<td>xanthine dehydrogenase</td>
<td>Bone resorption</td>
<td>0.79</td>
<td>0.25</td>
</tr>
<tr>
<td>Ncdn</td>
<td>neurochondrin</td>
<td>Bone resorption</td>
<td>0.09</td>
<td>0.48</td>
</tr>
<tr>
<td>Creb3l1</td>
<td>camp responsive element binding protein 3-like 1</td>
<td>Bone resorption</td>
<td>0.53</td>
<td>0.23</td>
</tr>
<tr>
<td>Phospho1</td>
<td>phosphatase, orphan 1</td>
<td>Bone resorption</td>
<td>0.90</td>
<td>0.07</td>
</tr>
<tr>
<td>Ltbp3</td>
<td>latent transforming growth factor beta binding protein 3</td>
<td>Bone resorption</td>
<td>0.56</td>
<td>0.35</td>
</tr>
<tr>
<td>Ecm1</td>
<td>extracellular matrix protein 1</td>
<td>Bone resorption</td>
<td>1.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Grem1</td>
<td>gremlin 1</td>
<td>Bone resorption</td>
<td>0.72</td>
<td>0.47</td>
</tr>
<tr>
<td>Bmp4</td>
<td>bone morphogenetic protein 4</td>
<td>Bone resorption</td>
<td>0.60</td>
<td>0.30</td>
</tr>
<tr>
<td>Twist1</td>
<td>twist basic helix-loop-helix transcription factor 1</td>
<td>Bone resorption</td>
<td>0.65</td>
<td>0.052</td>
</tr>
<tr>
<td>Ptk2b</td>
<td>ptk2 protein tyrosine kinase 2 beta</td>
<td>Bone resorption</td>
<td>0.69</td>
<td>0.14</td>
</tr>
<tr>
<td>Bglap2</td>
<td>bone gamma-carboxyglutamate protein 2</td>
<td>Bone resorption</td>
<td>1.62</td>
<td>0.34</td>
</tr>
</tbody>
</table>
Figure 5.10. Normalisation boxplot showing all the data compensated for against batch effects to allow for comparisons.
Figure 5.11. Gene expression of Wt1 deletion mutant bone cells differs to the gene expression of WER control bone cells, CAGG-CreER\textsuperscript{TM} Cre\textsuperscript{+}, and Cre\textsuperscript{−} bone cells. A. The Principal Component Analysis (PCA). PC1 shows a difference of 50% in expression and PC2 shows a difference of 30% in expression. The mutant bone cells (BH M labelled red) are clustered furthest away from the other populations. B. The cluster dendrogram shows the same thing. The height signifies the difference in populations, with mutant bone cells (BH M) again being the outliers.
5.4. Gene expression validation

Validation was required of the interesting GO terms which were enriched in the total bone microarray, comparing control versus mutant (i.e. *Wt1* deletion) as well as normoxia versus hypoxia. qRT-PCR was carried out on custom plates for 91 genes relating to the various GO terms as well as 5 reference genes (in biological and technical triplicate). qRT-PCR was also carried out in duplicate for each gene using one sample taken from CAGG-CreER<sup>TM</sup>;*Wt1*<sup>+/+</sup>Cre<sup>+</sup> and Cre<sup>-</sup> mice to further assess the effects of CreER recombination activation, as well as comparing it to the expression data from the CAGG-CreER<sup>TM</sup>;*Wt1*<sup>+-/+</sup> microarray.

As there is only one sample for the CAGG-CreER<sup>TM</sup> qRT-PCRs, a judgment call was made using the one qRT-PCR data set in combination with the Cre<sup>+</sup> and Cre<sup>-</sup> microarray values, to determine whether the effect of *Wt1* deletion on gene expression is true. For the majority of the 91 genes CreER recombinase activation had no effect and so the change in expression was due to *Wt1* deletion; examples of these are shown in Figure 5.12. However, the results suggested that a small number of genes were being affected by CreER recombinase activation and so these genes were discarded from the study. Figure 5.13 shows examples of genes on which CreER recombinase was having an effect.

The microarray results showed several genes to be down regulated when *Wt1* was deleted in bone cells cultured under hypoxia (mutant) compared to control bone cells. These down regulated genes were enriched for several GO terms relating to mesenchymal biology. Genes from the following GO terms were validated by qRT-PCR: collagen biosynthetic process (Figure 5.14), cartilage development (Figure 5.15), muscle tissue development (Figure 5.16), positive regulation of EMT (Figure 5.17), positive regulation of Wnt signalling (Figure 5.18), angiogenesis regulation (Figure 5.19) and response to hypoxia (Figure 5.20, plus data for Smad3, Sox4, Tgfb2, Tgfb3, Vegfa, Cav1 shown in Figures 5.17, 5.18, and 5.19). As shown previously, genes up regulated after *Wt1* deletion enrich for inflammatory response GO terms and were also validated by qRT-PCR (Figure 5.21). Interestingly several of these inflammatory genes (not included in Figure 5.21) were affected by CreER activation as seen in Figure 5.13.
Figure 5.12. Examples of genes not affected by CreER activation. In each case the gene expression from the control bone cultured in hypoxia is comparable to the CAGG-CreER\textsuperscript{TM};Wt1\textsuperscript{+/+} Cre\textsuperscript{−} and Cre\textsuperscript{+} bone cultured under hypoxia, especially when compared to the Wt1 deletion mutant. There is also very little difference in the expression of Cre\textsuperscript{−} and Cre\textsuperscript{+} expression suggesting activated CreER recombinase is not having an effect. (control and mutant n=3, Cre\textsuperscript{−} and Cre\textsuperscript{+} n=1).
Figure 5.13. Examples of genes affected by CreER activation. In each case the gene expression from the control bone cultured in hypoxia is comparable to the CAGG-CreER<sup>TM</sup>;Wt1<sup>+/+</sup> Cre<sup>-</sup> but not the Cre<sup>+</sup> bone cultured under hypoxia. There is a large difference in the expression of Cre<sup>-</sup> and Cre<sup>+</sup> expression, similar to the Control and Mutant difference, suggesting activating CreER recombinase is having an effect. (control and mutant n=3, Cre<sup>-</sup> and Cre<sup>+</sup> n=1).
Figure 5.14. qRT-PCR validating the down regulation of genes associated with the collagen biosynthetic process GO term in Wt1 deletion mutant bone cells cultured in vitro. The expression of Arg1 is significantly reduced in Wt1 deletion mutants cultured under normoxia (** = p<0.01) and hypoxia (* = p<0.05). Col3a1 is reduced in mutants under normoxia (p<0.05). Col1a1 and Col5a1 are reduced in mutant cells cultured under both normoxia and hypoxia (p<0.05). Serpinh1 is reduced in mutants cultured under normoxia (p<0.01) and hypoxia (p<0.05). (n=3).
Figure 5.15. qRT-PCR validating the down regulation of genes associated with the cartilage development GO term in Wt1 deletion mutant bone cells cultured in vitro. The expression of Ddah1, Prrx1, Prrx2, Rarb, Zeb1, and Shox2 is significantly reduced in Wt1 deletion mutants cultured under hypoxia (* = p<0.05). Thbs1 expression is reduced in mutant cells under normoxia (p<0.05) and hypoxia (** = p<0.01). Col1a1 is reduced in mutant cells cultured under both normoxia and hypoxia (p<0.05). (n=3)
Figure 5.16. qRT-PCR validating the down regulation of genes associated with the muscle tissue morphogenesis GO term in Wt1 deletion mutant bone cells cultured in vitro. The expression of Fgfr2, Shox2, Ttn, Foxc1, and Foxs1 is significantly reduced in Wt1 deletion mutants cultured under hypoxia (* = p<0.05). Expression of Foxs1 is also reduced in mutant cells cultured under normoxia (p<0.05). Col3a1 is also reduced in mutant cells cultured under normoxia (p<0.05). (n=3).
**Figure 5.17.** qRT-PCR validating the down regulation of genes associated with the positive regulation of epithelial to mesenchymal transition GO term in Wt1 deletion mutant bone cells cultured in vitro. The expression of 1190002H23Rik, Col1a1, Tgfb3, and Smad3 is significantly reduced in Wt1 deletion mutants cultured under normoxia (* = p<0.05) and hypoxia (p<0.05). Expression of Snai1 is also reduced in mutant cells cultured under normoxia (** = p<0.001) and hypoxia (p<0.05). Tgfb2 is reduced in mutant cells cultured under hypoxia (p<0.001). Zeb1 is reduced in mutant cells cultured under hypoxia (p<0.05). (n=3)
Figure 5.18. qRT-PCR validating the down regulation of genes associated with positive regulation of Wnt signalling GO term in Wt1 deletion mutant bone cells cultured in vitro. The expression of Dvl1, Ror2, Fgfr2, and Cav1 is significantly reduced in Wt1 deletion mutants cultured under hypoxia (* = p<0.05 and ** = p<0.01). Expression of Col1a1, Smad3, and Sox4 is also reduced in mutant cells cultured under both normoxia and hypoxia (p<0.05). (n=3).
Figure 5.19. qRT-PCR validating the down regulation of genes associated with the angiogenesis regulation GO term in Wt1 deletion mutant bone cells cultured in vitro. (* = p<0.05, ** = p<0.01 and *** = p<0.001). (n=3).
Figure 5.20. qRT-PCR validating the down regulation of genes associated with response to hypoxia GO term in Wt1 deletion mutant bone cells cultured in vitro. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). (n=3).
Figure 5.21. qRT-PCR validating the up regulation of genes associated with the inflammatory response GO term in Wt1 deletion mutant bone cells cultured in vitro. (* = p<0.05, ** = p<0.01, *** = p< 0.001). (n=3).
5.5. **Lin^−^CD31^−^GFP sorted microarrays**

To investigate the GFP population, WGER marrow and bone cells were stained with the lineage panel (CD3e, CD11b, CD45R, Ly-6G and Ly-6C, TER-119) and CD31 endothelial marker and then cells were sorted into Lin^−^CD31^−^GFP^+^ and Lin^−^CD31^−^GFP^−^ populations before being analysed for gene expression levels. The cells were only cultured under hypoxia as this gave increased gene changes previously. These WGER cells (CAGG-CreER^{T2};Wt1^{loxP/loxP}) have the tamoxifen inducible Wt1 deletion allowing the comparison of control GFP^+^ cells with mutant (i.e. Wt1 deletion) GFP^+^ cells. This determined what changes in gene expression were caused by loss of Wt1 expression purely in the GFP^+^ population. In total 4 comparisons were made;

1) Control GFP^+^ vs. Mutant GFP^+^  
2) Control GFP^−^ vs. Mutant GFP^−^  
3) Control GFP^+^ vs. Control GFP^−^  
4) Mutant GFP^+^ vs. Mutant GFP^−^  

Again GOrilla and DAVID gene ontology analysis tools were used to identify enriched GO terms. Marrow cells cultured under hypoxia, for all 4 comparisons, showed no genes to be differentially expressed with a q value <0.05. This was not the case for the bone cells, which showed significant differentially expressed genes for all 4 comparisons, with the majority being between control GFP^+^ and mutant GFP^+^ cells.

5.5.1. **GO terms down regulated in Wt1 mutant GFP^+^ cells**

Differentially expressed genes were ordered by log fold change and analysed using GOrilla. Genes which were down regulated in the GFP^+^ Wt1 mutant bone cells were largely enriched for GO terms related to lipid synthesis and metabolism (Table 5.18 and Figure 5.22). RNA processing GO terms were also enriched in this gene set (Table 5.19 and Figure 5.23). Analysis in DAVID used genes which were differentially down regulated in the Wt1 mutant bone GFP^+^ cells. Usually a q value below 0.05 and a log fold change greater than 1.5 was used as a cut off, however in
this case no genes had a log fold change of this value. Despite this fact, when these genes were run in DAVID similar GO terms were enriched in this data set as was seen by GOrilla. These include mRNA processing, mRNA metabolic process, and mitotic cell cycle.

5.5.2. GO terms up regulated in \( Wt1 \) mutant GFP\(^+\) cells

Genes which were up regulated in the GFP\(^+\) \( Wt1 \) mutant bone cells were mainly enriched for immune response and apoptotic related GO terms (Table 5.20 and Figures 5.24 and 5.25). For analysis using DAVID, only 11 genes had a log fold change in expression greater than 1.5 and a q value below 0.05. Even so, these genes also showed enrichment in 9 GO terms, all immune related, including innate immune response, acute inflammatory response, immune effector process, and endocytosis.

5.5.3. GO terms enriched in \( Wt1 \) mutant GFP\(^-\) cells

Genes which were down regulated in the GFP\(^-\) \( Wt1 \) mutant bone cells were enriched for a mixture of GO terms, probably due to this being a heterogeneous population of cells. A selection of these GO terms is shown in Table 5.21 with one of the most interesting being ossification. This suggests that \( Wt1 \) deletion is down regulating genes involved in bone formation in the cells which do not express \( Wt1 \), presumably through a paracrine signalling pathway. However, as shown previously, GFP\(^-\) cells still express low levels of \( Wt1 \) and so deletion may actually be having a functional effect in these cells.

As with the GFP\(^+\) and total populations, the genes which were upregulated in the \( Wt1 \) mutant GFP\(^-\) bone cells were also enriched for immune response GO terms.

5.5.4. GO terms enriched in control GFP\(^+\) cells

When looking at what genes are differentially expressed in GFP\(^+\) bone cells compared to GFP\(^-\) bone cells only 6 genes are significant (Table 5.22). Comfortingly, eGFP is among these 6 and is the most significantly upregulated gene in the GFP\(^+\) cells. GOrilla takes into account all differential expression changes.
irrespective of significance, and analysis showed that genes up regulated in the GFP\(^+\) population are enriched for several sterol and lipid related terms as well as other interesting terms (Table 5.23 and Figure 5.26).

GO terms enriched in the down regulated genes from the GFP\(^+\) compared to GFP\(^-\) bone cells are various immune responses and immune system processes. Bone mineralisation is also enriched with a q value of 1.95 \(\times\) 10\(^{-3}\) suggesting that bone mineralisation genes are more highly expressed in the GFP\(^-\) population. Again this adds to the evidence of paracrine interactions between the GFP\(^+\) and GFP\(^-\) populations.

Table 5.18. Enriched GOrilla GO terms of genes down regulated in GFP\(^+\) Wt1 mutant bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006629</td>
<td>Lipid metabolic process</td>
<td>20</td>
<td>3.08</td>
<td>1.03 (\times) 10(^{-4})</td>
</tr>
<tr>
<td>GO:1901617</td>
<td>Organic hydroxyl compound biosynthetic process</td>
<td>12</td>
<td>14.88</td>
<td>3.70 (\times) 10(^{-7})</td>
</tr>
<tr>
<td>GO:0006694</td>
<td>Steroid biosynthetic process</td>
<td>11</td>
<td>19.10</td>
<td>1.62 (\times) 10(^{-7})</td>
</tr>
<tr>
<td>GO:0016125</td>
<td>Sterol metabolic process</td>
<td>12</td>
<td>17.63</td>
<td>6.17 (\times) 10(^{-8})</td>
</tr>
<tr>
<td>GO:0016126</td>
<td>Sterol biosynthetic process</td>
<td>13</td>
<td>28.27</td>
<td>2.16 (\times) 10(^{-11})</td>
</tr>
<tr>
<td>GO:0006695</td>
<td>Cholesterol biosynthetic process</td>
<td>11</td>
<td>29.90</td>
<td>3.37 (\times) 10(^{-10})</td>
</tr>
</tbody>
</table>
**Figure 5.22.** Lipid metabolism related GO terms shown by the GOrilla visualization tool to be down regulated in mutant GFP<sup>+</sup> bone cells

**Table 5.19.** Enriched GOrilla GO terms of genes down regulated in GFP<sup>+</sup> Wt1 mutant bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0008380</td>
<td>RNA splicing</td>
<td>52</td>
<td>2.90</td>
<td>1.95 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>GO:0006397</td>
<td>mRNA processing</td>
<td>42</td>
<td>2.71</td>
<td>1.19 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>GO:0006260</td>
<td>DNA replication</td>
<td>33</td>
<td>3.13</td>
<td>1.26 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>GO:0090304</td>
<td>Nucleic acid metabolic process</td>
<td>223</td>
<td>1.33</td>
<td>3.68 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>GO:0045787</td>
<td>Positive regulation of cell cycle</td>
<td>8</td>
<td>6.47</td>
<td>3.44 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>GO:0006396</td>
<td>RNA processing</td>
<td>69</td>
<td>2.17</td>
<td>4.50 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>GO:0006412</td>
<td>Translation</td>
<td>49</td>
<td>2.79</td>
<td>2.15 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Figure 5.23. RNA processing GO terms shown by the GOrrilla visualization tool to be down regulated in mutant GFP$^+$ bone cells.

Table 5.20. Enriched GOrrilla GO terms of genes up regulated in GFP$^+$ Wt1 mutant bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006955</td>
<td>Immune response</td>
<td>90</td>
<td>4.99</td>
<td>1.27 x 10$^{-33}$</td>
</tr>
<tr>
<td>GO:0006952</td>
<td>Defense response</td>
<td>103</td>
<td>3.86</td>
<td>6.91 x 10$^{-29}$</td>
</tr>
<tr>
<td>GO:0006954</td>
<td>Inflammatory response</td>
<td>41</td>
<td>3.76</td>
<td>1.37 x 10$^{-20}$</td>
</tr>
<tr>
<td>GO:0045087</td>
<td>Innate immune response</td>
<td>46</td>
<td>6.42</td>
<td>6.38 x 10$^{-20}$</td>
</tr>
<tr>
<td>GO:0010941</td>
<td>Regulation of cell death</td>
<td>114</td>
<td>2.00</td>
<td>7.40 x 10$^{-16}$</td>
</tr>
<tr>
<td>GO:0042981</td>
<td>Regulation of apoptotic process</td>
<td>110</td>
<td>2.05</td>
<td>4.19 x 10$^{-19}$</td>
</tr>
<tr>
<td>GO:0008285</td>
<td>Negative regulation of cell proliferation</td>
<td>73</td>
<td>2.24</td>
<td>3.65 x 10$^{-8}$</td>
</tr>
<tr>
<td>GO:0043066</td>
<td>Negative regulation of apoptotic process</td>
<td>63</td>
<td>2.43</td>
<td>4.36 x 10$^{-8}$</td>
</tr>
</tbody>
</table>
Figure 5.24. RNA processing GO terms shown by the GOrilla visualization tool to be down regulated in mutant GFP bone cells.

Figure 5.25. Apoptosis related GO terms shown by the GOrilla visualization tool to be down regulated in mutant GFP bone cells.
Table 5.21. Enriched GOrilla GO terms of genes down regulated in GFP Wt1 mutant bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006412</td>
<td>Translation</td>
<td>62</td>
<td>3.00</td>
<td>1.24 x 10^{-9}</td>
</tr>
<tr>
<td>GO:0016126</td>
<td>Sterol biosynthetic process</td>
<td>14</td>
<td>5.82</td>
<td>4.00 x 10^{-4}</td>
</tr>
<tr>
<td>GO:0016125</td>
<td>Sterol metabolic process</td>
<td>19</td>
<td>3.41</td>
<td>1.47 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0006695</td>
<td>Cholesterol biosynthetic process</td>
<td>10</td>
<td>5.20</td>
<td>1.88 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0001503</td>
<td>Ossification</td>
<td>10</td>
<td>5.68</td>
<td>3.83 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0006397</td>
<td>mRNA processing</td>
<td>45</td>
<td>1.89</td>
<td>4.39 x 10^{-2}</td>
</tr>
</tbody>
</table>

Table 5.22. Genes which are significantly differentially expressed in GFP+ bone cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Log Fold Change</th>
<th>q value (adjusted p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOC669053</td>
<td>-1.45</td>
<td>0.007</td>
</tr>
<tr>
<td>eGFP</td>
<td>1.54</td>
<td>0.014</td>
</tr>
<tr>
<td>S100a8</td>
<td>-4.87</td>
<td>0.026</td>
</tr>
<tr>
<td>S100a9</td>
<td>-3.97</td>
<td>0.026</td>
</tr>
<tr>
<td>Actc1</td>
<td>-1.83</td>
<td>0.026</td>
</tr>
<tr>
<td>Pdk4</td>
<td>-1.03</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Table 5.23. Enriched GOrilla GO terms of genes up regulated in GFP+ bone cells cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>Number of Genes</th>
<th>Enrichment</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0016126</td>
<td>Sterol biosynthetic process</td>
<td>11</td>
<td>32.05</td>
<td>5.44 x 10^{-10}</td>
</tr>
<tr>
<td>GO:0016125</td>
<td>Sterol metabolic process</td>
<td>14</td>
<td>15.69</td>
<td>7.10 x 10^{-9}</td>
</tr>
<tr>
<td>GO:0006694</td>
<td>Steroid biosynthetic process</td>
<td>12</td>
<td>15.89</td>
<td>2.01 x 10^{-7}</td>
</tr>
<tr>
<td>GO:0008203</td>
<td>Cholesterol metabolic process</td>
<td>12</td>
<td>14.57</td>
<td>4.24 x 10^{-7}</td>
</tr>
<tr>
<td>GO:0006695</td>
<td>Cholesterol biosynthetic process</td>
<td>11</td>
<td>13.18</td>
<td>6.87 x 10^{-7}</td>
</tr>
<tr>
<td>GO:0008610</td>
<td>Lipid biosynthetic process</td>
<td>15</td>
<td>6.36</td>
<td>6.66 x 10^{-5}</td>
</tr>
<tr>
<td>GO:0060548</td>
<td>Negative regulation of cell death</td>
<td>40</td>
<td>2.46</td>
<td>6.77 x 10^{-4}</td>
</tr>
<tr>
<td>GO:0043066</td>
<td>Negative regulation of apoptotic process</td>
<td>36</td>
<td>2.46</td>
<td>2.46 x 10^{-3}</td>
</tr>
<tr>
<td>GO:0014909</td>
<td>Smooth muscle cell migration</td>
<td>3</td>
<td>52.44</td>
<td>1.40 x 10^{-2}</td>
</tr>
<tr>
<td>GO:0045766</td>
<td>Positive regulation of angiogenesis</td>
<td>19</td>
<td>2.77</td>
<td>4.01 x 10^{-2}</td>
</tr>
</tbody>
</table>
As covered in the introduction chapter there is evidence that \textit{Wt1} may regulate chemokines in the developing heart and kidney (Velecela \textit{et al.}, 2013). These data showed that \textit{Wt1} regulates \textit{Cxcl10}, an important recruiter of inflammatory cells, directly, and indirectly via regulation of transcription factor IRF7, which in turn regulates chemokine \textit{Cxcl10}. \textit{Cxcl1}, another inflammatory recruiting chemokine involved in osteoclast and osteoblast formation (Onan \textit{et al.}, 2009), and \textit{Cxcl10} were investigated further as inflammation was a GO term often enriched in the set of up

\textbf{Figure 5.26. Loss of Wt1 expression causes chemokine genes, Cxcl1 and Cxcl10, to significantly increase expression in cultured bone marrow.} * = p<0.05 and ** = p<0.01 (control n=5, mutant n=7).
regulated genes following \textit{Wt1} deletion. Figure 5.26 shows that in bone marrow, cultured under both normoxia and hypoxia, deletion of \textit{Wt1} results in a significant increase in the expression of \textit{Cxcl10} and \textit{Cxcl1}. These qRT-PCRs were carried out on total cultured bone marrow cells; however when looking at Lin^-CD31^-GFP^+ and GFP^- sorted cells the microarray expression data show the same finding. When comparing control GFP^+ bone cells with mutant (i.e. \textit{Wt1} deletion) GFP^+ bone cells \textit{Cxcl1} expression was increased by a log fold change of 0.96 in the mutant (q value <0.05). The same result was seen in the GFP^- cells with a log fold increase of 0.89 in the mutant (q value <0.05). For \textit{Cxcl10} the microarray data show the same increased expression trend in the mutant but the q values were not significant (q=0.41). This may be due to the GFP population being split from the GFP^- or it may be that the microarray data are not as stringent and reliable as qRT-PCR results. Either way, the loss of \textit{Wt1} is having an effect on inflammatory genes and chemokines.

5.6. Discussion

The results from the total cell microarrays shed light on what genes are affected in the population as a whole. Although relatively few genes were differentially expressed in the marrow, too few to result in GO term enrichment, they were still relevant genes. The loss of \textit{Wt1} expression caused a reduction in \textit{Atf4} expression, which codes for activating transcription factor 4 (ATF4). ATF4 is vital for bone homeostasis as it is a regulator of osteoblast and osteoclast differentiation from bone marrow derived MSCs (Yu \textit{et al.}, 2013). Knocking out \textit{Atf4} impairs the ability of MSCs to differentiate into osteoblasts \textit{in vitro}, whilst knocking out \textit{Atf4 in vivo} has a significant negative effect on bone mass and formation (Yang \textit{et al.}, 2004; Yu \textit{et al.}, 2013). The reduction of \textit{Atf4} seen in the \textit{Wt1} mutant marrow cells would suggest it is down stream of \textit{Wt1} and is in some way responsible for the loss of bone mass seen in the \textit{in vivo} \textit{Wt1} knockout mice. The microarray highlights an increase in \textit{Eif3f} expression, which codes for the translation initiation factor eIF3 which positively regulates the Notch pathway by deubiquitinating Notch and allowing it to enter the nucleus (Moretti \textit{et al.}, 2010). Notch signalling maintains mesenchymal progenitors and regulates chondrocyte proliferation and osteoblast differentiation, making it
important for bone homeostasis and maintaining the growth plate (Hilton et al., 2008). It does this by indirectly inhibiting Runx2 through Hes1, Hey1, and HeyL proteins (Hilton et al., 2008). Here we show that following Wt1 deletion Eif3f was increased which will result in increased Notch signalling, therefore disrupting the bone homeostatic balance by inhibiting osteoblast differentiation, again resulting in a decreased bone mass as seen in the in vivo Wt1 knockout mice.

Wt1 seems to have a significant role in the bone cells as several thousand genes were affected by the loss of its expression; a large enough set of genes for various GO terms to be enriched. Loss of Wt1 expression negatively affects GO terms related to the developmental processes of the mesoderm, cartilage, blood vessels, and skeletal system. All these processes are systems which derive from mesenchymal cells, suggesting that Wt1 is playing a significant functional role in the differentiation and development of various mesenchymal tissues, including the bone. Interestingly, loss of Wt1 expression also negatively affects the VEGF receptor signalling pathway, angiogenesis regulation, and the canonical Wnt receptor signalling pathway. These are less surprising as Wt1 is known to be involved with these processes as are MSCs (Karth et al., 2000; Davies et al., 2004). On the other hand, the loss of Wt1 expression is having a positive effect on various immune responses, which is not surprising considering the number of negatively affected tissue processes.

When hypoxia and normoxia were compared there were only a small number of enriched GO terms. Unsurprisingly, responses to oxygen levels and hypoxia were increased in the hypoxic cells compared to normoxic, but the glycolysis GO term had the highest enrichment. Glycolysis is used as an adaptive strategy by tumour cells for the survival of hypoxic cells within the tumour and for the growth of normoxic cancer cells (Guillaumond et al., 2013). Glycolysis in tumours is usually associated with tumour survival and aggressiveness (Guillaumond et al., 2013).

Various immune response GO terms were down regulated in the hypoxic bone cells compared with the normoxic, but also bone remodelling and bone resorption terms. The hypoxic nature of the bone marrow stem cell niche is vital for maintaining the quiescence of stem cells. In increased oxygen levels, found in close proximity to the bone and its vasculature, cells lose their quiescence and are more likely to
differentiate (Fehr 

\textit{et al.}, 2007; Mohyeldin \textit{et al.}, 2010). Therefore, it is not unexpected that under hypoxia bone remodelling is reduced as this is when the cells are most quiescent.

In the previous chapter I showed data to strongly suggest that activation of CreER recombinase has an effect on the colony forming ability of bone and marrow cells. However, the microarray results from CAGG-CreER\textsuperscript{TM};\textit{Wt1}\textsuperscript{+/+} cells (lacking \textit{loxP} sites) were less dramatic with only 15 genes being significantly altered by CreER activation. Bone resorption and bone remodelling GO terms were shown to be up regulated in the Cre\textsuperscript{+} bone cells. This initially suggests that the bone resorption and increased osteoclast numbers observed following \textit{Wt1} deletion \textit{in vivo} may be due to the activation of CreER recombinase rather than the loss of \textit{Wt1} expression. However, after further thought and investigation there are several factors which suggest this enrichment may not be true. Firstly, the results from this microarray are carried out on bone cells cultured \textit{in vitro} for 7 days and are therefore not 100\% comparable to results found \textit{in vivo}. Secondly, although the GO terms themselves are considered statistically up regulated by GOrilla, they are in fact made up of 20 genes that are not significantly up regulated. Thirdly, when the gene set is ordered by statistical significance rather than log fold change in the GOrilla tool these GO terms are no longer enriched. Fourth and finally, principal component analysis and a cluster dendrogram on the normalised data visually show the \textit{Wt1} mutant bone cells to be outliers when compared to the other data sets and the CAGG-CreER positive;\textit{Wt1}\textsuperscript{+/+} cells are not. To my mind, this renders these GO terms unconvincing and unreliable, but to fully verify this qRT-PCRs will be carried out in future work.

The question does arise, however, as to why CreER recombinase is having such a prominent effect on colony formation and yet a relatively small, if any, effect on gene expression. There is also an absence of genes one might expect to see differentially expressed, such as proliferation genes, which would complement the phenotypes seen from the CFU-F assays. The culture conditions and protocol differs for these two experiments and so could be a starting place to address this question.

Enriched GO terms were validated using custom qRT-PCR plates with 91 genes of interest on them. Genes from 7 GO terms were shown to be down regulated in \textit{Wt1}
deletion mutants and genes from the inflammatory response GO term were up regulated. The first three of these GO terms are collagen biosynthetic process, cartilage development, and muscle tissue development; processes involving mesenchymal tissues maintained by MSCs and their progenitors. This is again reinforcing the functional involvement of Wt1 in this population. The fourth and fifth GO terms, positive regulation of EMT and positive regulation of Wnt signalling, are known to be dependent on Wt1 expression during development. EMT has only been observed during development; however these novel data show the functional importance of Wt1 expression on genes involved in adult EMT. This is a new and exciting finding and could lead to better understanding whether EMT plays a role in adult tissue homeostasis.

The final two GO terms which were validated are angiogenesis regulation and response to hypoxia. These two terms are already known to be linked with Wt1 expression via VEGF and HIF1α proteins. The data here confirm this and show that these genes interact in the bone cell population. One gene of interest which validates the hypoxia GO term is Egln3 which codes for hypoxia-inducible factor prolyl hydroxylase 3. Proline hydroxylation is used to degrade and destroy HIF, usually in the presence of oxygen (Ivan et al., 2001), therefore Egln3 is involved in weakening HIF activity. However, in humans HIF has been shown, via hypoxic induction, to regulate this process by binding to a HRE sequence on the EGLN3 gene and driving transcription forming a negative feedback loop (Pescador et al., 2005). Wagner et al. (2003) showed that Wt1 expression is stimulated by HIF-1, also by its binding to the HRE of the Wt1 promoter. The fact that Wt1 deletion causes a significant reduction in Egln3 expression suggests that Egln3 may be a target of Wt1, and as HIF-1 activates Wt1, may also be acting in this negative feedback loop.

qRT-PCRs were also carried out on samples from CAGG-CreER<sup>T2</sup>;Wt1<sup>+/+</sup> mice, Cre<sup>+</sup> and Cre<sup>-</sup>, to assess for the effects of activated CreER recombinase. A minimal number of genes were affected and were mainly found to be genes in the inflammatory GO terms up regulated in Wt1 deletion mutants. The genes were removed from the analysis and the inflammatory GO term still remained enriched.
When microarrays were carried out on the sorted cell populations (i.e. Lin\(^{-}\)CD31\(^{-}\) GFP\(^{+}\) and GFP\(^{-}\)) lipid synthesis, lipid metabolism, and RNA processing GO terms were down regulated in the GFP\(^{+}\) \textit{Wt1} deletion mutant bone cells compared to the GFP\(^{+}\) control cells. This suggests that \textit{Wt1} has a functional role in the ability of GFP\(^{+}\) cells to form lipids, which is also a characteristic of adipocytes. In Chapter 4, Figures 4.1 and 4.2, preliminary studies suggest that GFP\(^{+}\) cells are capable of adipocyte differentiation. As well this, recent work by Chau \textit{et al.} (2014) shows that adipocyte progenitors express \textit{Wt1} in the visceral fat pads. Therefore the down regulation of lipid synthesis genes is not too surprising but offers new genes to explore the mechanisms involved.

When the remaining GFP\(^{-}\) population was compared, between control and \textit{Wt1} mutant, the genes down regulated in \textit{Wt1} mutant bone cells were enriched for a variety of GO terms, one of the most interesting being ossification. The fact that \textit{Wt1} has a functional effect on ossification genes, but only in GFP\(^{-}\) cells, suggests that \textit{Wt1} is acting via paracrine signalling with regard to bone formation.

The last comparison was between the control GFP\(^{+}\) bone cells and the control GFP\(^{-}\) bone cells. There seems to be little difference between the two populations with only 6 genes changing significantly; 1 up in the GFP population, unsurprisingly \textit{eGFP}, and 5 down. The downregulated genes include \textit{S100a8} and \textit{S100a9} which code for calcium binding proteins involved in autoimmunity and cancer development; \textit{Actc1} which codes for cardiac muscle alpha actin protein which is important for contraction of the cardiac muscle tissue; and \textit{Pdk4} which codes for a pyruvate dehydrogenase kinase involved in glucose and fatty acid metabolism. When these differentially expressed genes were analysed in GOrilla several sterol, cholesterol, and lipid metabolism and biosynthesis GO terms were enriched in the GFP\(^{+}\) population compared to the GFP\(^{-}\). The fact that the GFP\(^{+}\) and GFP\(^{-}\) populations show little variation in gene expression may be due to \textit{Wt1} being expressed at low levels in all cells, but being highest in the GFP\(^{+}\) cells. It is also possible that the expression may be cycling, or that \textit{Wt1} expression may be having a systemic paracrine effect on both GFP\(^{+}\) and GFP\(^{-}\) populations. Another question which arises is why so few genes are differentially expressed in the GFP sorted populations when \textit{Wt1} is deleted,
compared to the total cell populations. These are questions that will be addressed in the discussion and future work chapter.

The final data section in this chapter looked at the effect of Wt1 deletion on increasing inflammatory processes and chemokines. Various inflammatory and immune GO terms were increased in the CAGG-CreER positive; Wt1<sup>loxP/loxP</sup> mutant cells and 9 genes involved in these processes were validated by qRT-PCR, as well as the increased expression of chemokines Cxcl1 and Cxcl10. These data back up the involvement of Wt1 in inflammation and concur with the findings of Velecela et al. (2013) who showed that Wt1 regulates Cxcl10 in kidney and heart, which up until now has not been shown to be the case in the bone.
Chapter 6

*Wt1* expression during bone development
6.1. Introduction

Wt1 expression is vital during development with homozygote mutants resulting in embryonic lethality at around embryonic day 13-15 (e13-e15) (Kreidberg et al., 1993). Failure of these mutants to survive is due to the defective development of several organs; mainly the heart, but also kidneys, lungs, diaphragm, and gonads. Wt1 is essential for kidney formation and is expressed in the kidney throughout development. It is also expressed in other developing organs including gonads, spleen, and mesothelium, but is first expressed at e9.0 in the intermediate mesenchyme (which develops to form the kidneys and gonads) (Armstrong et al., 1993). At e9.5, Wt1 expression is observed in the septum transversum mesenchyme which is the source of the epicardium, the diaphragm, hepatic sinusoids, and mesothelium of the liver (Chau and Hastie, 2012). Wt1 then continues to be expressed in various tissues throughout development (See Chapter 1 Introduction for further details). However, the expression of Wt1 has not been fully characterised in the developing limb. Wt1 expression continues from development through to adulthood in some tissues, for example the kidneys, and as I have observed Wt1 expression in the adult bone and marrow, it was only logical to investigate expression in the developing bone and hypertrophic chondrocytes also.

6.2. GFP positive cells are present in developing limbs

The first question to be addressed was whether Wt1 was expressed in the developing limbs. To do this I used the Wt1-GFP mice; GFP protein expression is driven by the Wt1 promoter (See Chapter 3, Figure 3.1). Figure 6.1 shows that GFP, and therefore Wt1, is expressed in the hindlimb during development from e11.5 to e16.5. From e11.5 to e13.5 the hindlimb bud as a whole was analysed whilst at e16.5 it was possible to separate the cartilage and bone. Interestingly, expression seems to decrease as development progresses, with the lowest percentage being in the bone of e16.5 mice.

Once Wt1 expression was confirmed in the developing limb the location was the next question to be addressed. Figure 6.2 shows immunohistological sections of e11.5
mice. GFP and Wt1 expression was seen in the mesothelium which is a layer of cells that covers the internal organs (Mutsaers, 2004). However, no obvious expression was seen in the hindlimb buds despite over 4% of hindlimb cells being shown to express GFP (Figure 6.1). Dual immunofluorescence staining was also used at e11.5 in the limb bud (Figure 6.3). Upon close inspection the majority of staining was likely to be auto-fluorescence as it looked brighter even in the DAPI channel. However, amongst the auto-fluorescence it is possible that there is some true expression.

The metatarsal bones of the e14.5 hindlimb were then looked at (Figure 6.4). GFP staining was present in the growth plate area of the metatarsal bones but corresponding Wt1 staining was not seen. However, both Wt1 and GFP staining were again seen in the mesothelium of the lung cavity (Fig. 6.4 E and F) suggesting the antibodies are working well as these are used as positive controls. Immunofluorescence staining did not highlight expression of either Wt1 or GFP in the metatarsal bones but potentially some cells just outside and between the bones were positive. Again there was a lot of auto-fluorescence as is shown by the bright DAPI signal (Fig. 6.5 B) and the secondary only control (Fig. 6.5 D).

At e15.5 some interesting staining was observed in the metatarsal bones of the hindlimb. Cells along the inner bone surface, in between the bone and hypertrophic chondrocytes (which will become the marrow cavity), stained positively for Wt1 under both immunohistological DAB staining (Figure 6.6) and immunofluorescence staining (Figure 6.7 E and F). This was not seen at the other embryonic stages, including e14.5 and e16.5. Again the immunofluorescence showed some interdigital GFP staining (Fig. 6.7 A and B) which appears to be real when compared with the autofluorescence seen in the secondary only control. However, this was not corroborated with by any Wt1 staining.

As just mentioned, at e16.5 there was no Wt1 staining seen in the metatarsal bones (Fig. 6.8 A and B). Wt1 staining validity was confirmed by the positive control staining of the kidney glomerulus (Fig. 6.8 C and D) as well as the Wt1 positive mesothelial layer. The GFP stain, on the other hand, is present in the hypertrophic chondrocytes, growth plate, and bone terminus according to the
immunohistochemistry in Figure 6.9. The dual immunofluorescence suggests that there may be some interdigital expression of both Wt1 and GFP, however it is hard to determine whether this is true staining due to the secondary only control (Fig. 6.10 D) showing similar expression patterns.

![Figure 6.1. The mean percentage (±SEM) of total cells, analysed by flow cytometry, which are GFP⁺ decreases as development progresses from e11.5 to e16.5. (e11.5 and e16.5 n=3, e12.5 and e13.5 n=1).](chart.png)
Figure 6.2. GFP and Wt1 DAB immunohistochemistry on e11.5 sections. GFP (A&C) and Wt1 (B&D) are expressed (brown) in the mesothelial cells at e11.5. (see arrows). E and F are secondary only controls for GFP and Wt1 staining respectively. G is a GFP negative mouse as control for GFP staining. (scale bars A,B,G = 200 µm, C-F = 100 µm). (n=3).
Figure 6.3. GFP (green) and Wt1 (red) immunofluorescence on e11.5 sections. **A.** The hindlimb bud at e11.5. The split screen image (B) suggests that much of the staining may be auto-fluorescence. **C** and **D** are secondary only controls. (scale bars A&C = 200 µm, B&D = 50 µm). (n=3).
Figure 6.4. GFP and Wt1 DAB immunohistochemistry on e14.5 sections. **A** shows GFP staining (brown) in the hindlimb with **C** showing GFP expression in the cartilaginous cells of the metatarsal bone. **B** and **D** on the other hand show no Wt1 expression in the hindlimb metatarsal bones. **E** and **F** show GFP and Wt1, respectively, expressed in the mesothelial cells of the lung cavity. **G** and **H** are secondary only controls for GFP and Wt1 staining respectively. **I** is a GFP negative control for GFP staining. (scale bars A,B,H,I = 200 µm, C-G = 100 µm). (n=3).
Figure 6.5. GFP (green) and Wt1 (red) immunofluorescence on e14.5 sections. A. The hindlimb bud at e14.5 shows some GFP and Wt1 expression. The split screen image (B) suggests that some staining may be auto-fluorescence. C shows some true expression highlighted by arrows. D shows secondary only controls. (scale bars A = 250 µm, B-D = 50 µm). (n=3).
Figure 6.6. Wt1 DAB immunohistochemistry on e15.5 sections. A-E shows Wt1 staining (brown) in cells at the inner surface of the bone, between the bone and hypertrophic chondrocytes. F and G are secondary only controls for Wt1 staining. (scale bars A,C,G = 200 µm, B,D-F = 100 µm). (n=3).
Figure 6.7. GFP (green) and Wt1 (red) immunofluorescence on e15.5 sections. A. The hindlimb bud at e15.5 shows some GFP expression. B shows that it is between the metatarsal bones. C and D are secondary only controls. E and F show Wt1 expression in the cells along the inner surface of the bone (scale bars A&C = 250 µm, E&F = 200 µm, B&D = 50 µm). (n=3).(HC=hypertrophic chondrocytes).
Figure 6.8. Wt1 DAB immunohistochemistry on e16.5 sections. A and B show no Wt1 staining (brown) in the hindlimb. C and D show the positive control Wt1 staining of the podocytes in the kidney. E shows Wt1 staining in the mesothelium. F is a secondary only control for Wt1 staining. (scale bars A,C-F = 100 µm, B = 200 µm). (n=3).
Figure 6.9. GFP DAB immunohistochemistry on e16.5 sections. A and B show GFP staining (brown) in the hindlimb cartilaginous cells. C is a secondary only control. D shows GFP staining in the hind limb bone, mainly in the bone end (F) and cartilaginous growth plate (G). E is a secondary only control for GFP staining. (scale bars A,C-E = 500 µm, B,F,G = 100 µm). (n=3).
Figure 6.10. GFP (green) and Wt1 (red) immunofluorescence on e16.5 sections. A. GFP and Wt1 staining in hindlimb. B shows the interdigital region between the metatarsal bones. C&D are secondary only controls. E. Positive control showing Wt1 and GFP staining in the kidney glomerulus. F. is a kidney secondary only control (scale bars A&C = 250 µm, B,D-F = 50 µm). (n=3). (HC=Hypertrophic Chondrocytes).
6.3. The effect of \textit{Wt1} deletion on bone development

To look at \textit{Wt1}’s functional role in bone development it was deleted using the tamoxifen inducible CAGG-CreER^T2;\textit{Wt1}^{loxP/loxP} model. Maternal mice were gavaged with tamoxifen at e14.5 and the hind legs of the offspring were analysed at e18.5. Total cells from the developing limb were stained with the lineage panel markers (CD3e, CD11b, CD45R, Ly-6G and Ly-6C, TER-119) and CD31 endothelial marker to identify the haematopoietic fraction. The cells were also stained with the three MSC cell surface markers (CD29, CD73, and CD105) in order to relate the non-haematopoietic (i.e. Lin'CD31') MSC population (Triple$^+$) to the GFP positive population (GFP$^+$). This is a preliminary experiment and there are only 2 samples so far, therefore conclusive statistics were unable to be calculated. However, Figure 6.11 shows that deletion of \textit{Wt1} possibly has an effect on the non-haematopoietic proportion of cells as the percentage rises by just over 3%. Figure 6.12 also suggests that loss of \textit{Wt1} expression negatively affects the percentage of total cells which are non-haematopoietic MSCs (i.e. Lin'CD31'Triple$^+$), dropping from 0.08% to 0.007%. These preliminary data suggest that \textit{Wt1} plays a functional role in this non-haematopoietic MSC population and this will be followed up with a greater sample set.

Hematoxylin and eosin staining was also carried out using the second hindlimb of these \textit{in utero} deletion mice to assess any obvious morphological effects. Figure 6.13 shows images of the growth plate, joint, and marrow, for both control mice and \textit{Wt1} deletion mutants. The images suggest that no obvious morphological effect occurred. If loss of \textit{Wt1} expression is having a physical effect it may be too subtle to pick up by eye in the time frame of this experiment.
Figure 6.11. The mean percentage of total e18.5 hindlimb cells which are non-haematopoietic (i.e. Lin−CD31−) increases following the deletion of Wt1. (n=2).

Figure 6.12. The mean percentage of e18.5 total hindlimb cells which are non-haematopoietic MSCs (i.e. Lin−CD31− Triple+) decreases following the deletion of Wt1. (n=2).
Figure 6.13. Hematoxylin and eosin staining of e18.5 hindlimb sections shows no obvious difference between control mice and Wt1 deletion mice (mutant). A and B: Sections of the growth plate. C and D: Sections of the bone joint. E and F: Sections of the marrow cavity. (Scale bar = 100 µm).
6.4. Osteoblast progenitors during development

To assess whether cells from the developing limb express osteoblast progenitor markers flow cytometry analysis was carried out. Total hindlimb cells from e11.5 and e13.5 mice plus cartilage and bone from e16.5 mice were stained for the lineage panel markers and CD31 endothelial marker to again identify the haematopoietic fraction, however it may be too early to identify HSC and other haematopoietic cells at these developmental stages. The cells were also stained with Sca-1 and CD166 in order to relate the non-haematopoietic (i.e. Lin-CD31-) population to the osteoblast progenitors. As previously discussed in Chapter 3 (Section 3.4) Nakamura et al. (2010) have characterised these adult non haematopoietic cells by their expression of Sca-1 and CD166 cell surface markers. It is worth bearing in mind that these markers may not directly relate to osteoblast progenitors during development. The CD166-Sca-1- and CD166+Sca-1- populations were enriched for osteoblasts and had osteogenic potential. This is the first study to look at these populations during development and preliminary data suggests that the majority of hindlimb cells are found in these osteoblast progenitor populations (Figure 6.14). The data also suggest that as development progresses there may be a shift from the CD166-Sca-1- to the CD166+Sca-1- population. Both are capable of osteogenesis, however the ability to form adipocytes changes between these populations from what Nakamura et al., 2010 have shown; it is possible that this is of importance during development. On the other hand, it may be that CD166 levels are increasing due to cellular differentiation throughout development. These are preliminary data with very small sample sets and so further experiments are needed to verify this.

To assess whether \( Wt1 \) is playing a role in the osteoblast progenitor population \( Wt1 \) was deleted using the CAGG-CreER\(^T2\);\( Wt1^{loxP/loxP} \) mice. Maternal mice were gavaged with tamoxifen at e14.5 and hindlimb samples analysed at e18.5. Figure 6.15 suggests that \( Wt1 \) deletion was having little effect on the distribution of these cells. These are preliminary data with a sample size of 2 and so statistics are unable to be carried out reliably, however results at this stage suggest that there may be a significant increase in the percentage of Sca-1-CD166- cells, which as described by Nakamura et al. (2010) are the population of immature MSCs. Again, these results need to be repeated with an increased sample size to verify this trend. These findings
only look at the effect of \textit{Wil} on the distribution of these cells across the four quartiles, it does not determine whether \textit{Wil} has a functional role or not. Future experiments would include identifying which quartile the GFP\textsuperscript{+} cells are found in and sorting these populations to assess their differentiation capabilities.
**Figure 6.14.** Limb cells during development mainly express markers for osteoblast progenitors. Mean percentage of total cells which are either positive or negative for Sca-1 and CD166 markers. (e11.5 n=2, e13.5 and e16.5 n=1).

**Figure 6.15.** In utero deletion of Wt1 seems to have little effect on the mean percentage of cells expressing osteoblast progenitor markers (A). However there is a potentially significant increase in the percentage of Sca-1+CD166+ cells (B). (n=2).
6.5. Lineage tracing during development

In Chapter 4 (Section 4.6) I discussed the lineage tracing model \((Wt1^{CreERT2};mTmG}\) where Tomato is ubiquitously expressed under a pCA promoter, but following Cre mediated loxP recombination and the removal of Tomato, GFP is expressed. This allows cells with activated CreER recombinase (driven by the \(Wt1\) promoter) to be “tattooed” at the time of tamoxifen induction. These GFP-expressing “tattooed” cells can then be traced, as well as their progeny, to determine whether \(Wt1\) positive cells give rise and contribute to particular cells and tissues. Technical difficulties were encountered in the adult mice as discussed in Chapter 4. \textit{In utero} induction via maternal gavaging at e12.5 was carried out in the hope that the earlier the mice were induced the more chance of successful lineage tracing. Pups were analysed at e18.5, 10 days, and 3 weeks.

The hindlegs were embedded for paraffin sections and stained with GFP and Wt1 antibodies. A GFP positive cell which is not Wt1 positive will have originated from a Wt1 positive cell. Wt1 and GFP staining was carried out on the e18.5, 10 day, and 3 week mice and showed that staining was quite unspecific. Figure 6.16 shows the e18.5 growth plate, rich in chondrocytes, was awash with brown GFP and Wt1 DAB staining, even in the Cre samples which should be negative controls for GFP. Dual immunofluorescence of these two antibodies failed to highlight any positive cells in the growth plate/limb end region (Figure 6.16 G). The same unspecific staining, highlighted by expression in the Cre samples, was also seen in the day 10 (Figure 6.17) and 3 week (Figure 6.18) samples. Upon further in-depth investigation some convincingly positive GFP cells (with clean secondary only and Cre controls) and positive Wt1 cells were observed as outlined below.

6.5.1. Induction at e14.5 and analysis at e18.5

Figure 6.19 shows that hindlimb samples from the e18.5 mice express GFP in long flattened cells located at the edge of the bone surface. Dual immunofluorescence (GFP and Wt1 antibodies) also showed this bright GFP signal in the flattened cells running in close parallel proximity to the periosteum (Figure 6.20). Secondary only
controls and CRE− controls show that this staining is real. These cells were also negative for Wt1 staining (red) suggesting that they originate from a Wt1 expressing cell.

Using the Z-stack function of the confocal imaging suite these cells were cross sectioned three dimensionally. This allowed me to confirm that these GFP⁺ structures were indeed cells which contained a nucleus and had long thin processes (Figure 6.21).

6.5.2. Induction at e14.5 and analysis at 10 days

Figures 6.22 and 6.23 show that there are GFP positive cells in the hindlimbs analysed at 10 days. In Figure 6.22, the cells again have a thin and long morphology and are located close to the bone surface. Immunofluorescence highlighted similar long thin cells as well as some rounder clusters of cells in the tissue surrounding the bone (Figure 6.23).

Only a few cells were found to be GFP positive which is interesting as you would expect GFP⁺ cell numbers to increase in relation to the increased time from the initial induction of the lineage tracing model. You would therefore expect to see more GFP⁺ cells at day 10 than when analysed at e18.5, but this does not seem to be the case.

6.5.3. Induction at e14.5 and analysis at 3 weeks

Other than the unspecific staining of Wt1 and GFP mentioned earlier no positive cells were found along the bone surface as seen in the e18.5 and day 10 samples both also induced at e14.5. Flow cytometry analysis was also carried out on the bone and marrow of one mouse induced at e14.5 and analysed at 3 weeks and did not identify any GFP⁺ cells. This is consistent with the lack of GFP staining found following the immunohistochemistry, however this needs to be repeated to be conclusive.
Figure 6.16. Lineage tracing in the leg induced at e14.5 and analysed at e18.5. A-F are DAB immunohistochemistry images showing unspecific positive staining (brown) present in cells of the bone joint end and growth plate (scale bar = 200 µm). Images G and H are dual immunofluorescence images showing no GFP (green) or Wt1 (red) staining in the bone terminus or growth plate (scale bar = 200 µm). (n=3).
Figure 6.17. Lineage tracing in the leg induced at e14.5 and analysed at 10 days. DAB immunohistochemistry images showing unspecific positive staining (brown) present in cells of the bone terminus and growth plate (A-E scale bar = 200 µm, F = 100 µm). (n=3).
Figure 6.18. Lineage tracing in the leg induced at e14.5 and analysed at 3 weeks. DAB immunohistochemistry images showing unspecific positive staining (brown) present in cells of the bone growth plate (A-D scale bar = 200 µm, E = 100 µm). (n=3).
Figure 6.19. Lineage tracing in the leg induced at e14.5 and analysed at e18.5. A-E are DAB immunohistochemistry images showing GFP\(^{*}\) staining (brown) present in cells along the bone edge (scale bar A-C = 50 µm, D and E = 20 µm). Image F is a secondary only control (scale bar = 100 µm). (n=3).
Figure 6.20. Lineage tracing in the leg induced at e14.5 and analysed at e18.5. A and B are immunofluorescence images showing GFP<sup>+</sup> staining (green) present in cells along the bone edge. Image C is a secondary only control and image D is a Cre negative control. DAPI = blue and Wt1 = red (scale bar A and C = 200 µm, B and D = 50 µm). (n=3).
Figure 6.21. Lineage tracing in the leg induced at e14.5 and analysed at e18.5. A and B are Z-stack confocal images showing GFP⁺ immunofluorescence staining (green). The cross-sectional view is shown for the cell highlighted with an orange cross. DAPI = blue and Wt1 = red (scale bar = 50 µm).
Figure 6.22. Lineage tracing in the leg induced at e14.5 and analysed at 10 days. A and B show GFP\(^{+}\) staining (brown) present in cells lining the bone surface. Image F is a secondary only control and image G is a Cre negative control. (scale bar A,D,F,G = 50 µm, B,C,E = 20 µm). (n=3).
Figure 6.23. Lineage tracing in the leg induced at e14.5 and analysed at 10 days. A and B are immunofluorescence images showing GFP⁺ staining (green) present in cells next to the bone (bone shown by arrows). The area of interest highlighted by the white square in A is shown in image B. Image C is a secondary only control and image D is a Cre negative control. DAPI = blue and Wt1 = red (scale bar A = 250 µm, B = 50 µm, C and D = 200 µm). (n=3).
6.6. Discussion

The aim of this chapter was to determine whether $Wt1^+$ cells are found in the limb during development; the results suggest this to be the case. As discussed earlier $Wt1$ plays an important role in the development of various organs (Kreidberg et al., 1993) and these preliminary data are evidence to include developing bone and chondrocytes in this list. The flow cytometry data showed the presence of GFP$^+$ cells in the developing limb from e11.5 to e16.5. The percentage of GFP$^+$ cells seems to decrease across this time span suggesting that $Wt1$ expression may be more important for early development, perhaps for the differentiation of progenitors into osteoblasts and chondrocytes.

Using immunohistological techniques the location of these Wt1$^+$ and GFP$^+$ cells was investigated in the embryos with regard to developing hindlimbs; bone and cartilage. GFP and Wt1 expression was seen in the mesothelial cells at e11.5, e14.5, and e16.5. The mesothelium is a layer of cells which covers the internal organs and lines the internal cavities (Mutsaers, 2004). $Wt1$ is expressed in the mesothelium of the heart at e9.0, liver at e9.5, lung at e10.5, and gut at e11.5 (Armstrong et al., 1993; Walker et al., 1994; Wilm et al., 2005; Que et al., 2008; Chau and Hastie, 2012; Cano et al., 2013). However, compared with the mesothelial staining and the positive control staining of the glomerulus, there appeared to be very weak staining, if any at all, of GFP and Wt1 in the limb buds or the metatarsal bone and marrow. This is interesting as the flow cytometry results suggest that GFP$^+$ cells are present here from e11.5 to e16.5. There is a high amount of variation between Wt1 antibodies and their staining of developmental tissues. Optimisation of antibodies has shown that although all antibodies stain the kidney podocytes positively there is some variation in staining of the mesothelium depending on the antibody. By this logic there could also be variation in the staining of bone and hypertrophic chondrocytes. This antibody variation, combined with varying Wt1 expression levels, may be the reason for different staining patterns between the DAB immunohistochemistry and the immunofluorescence techniques, as well as between the GFP and Wt1 DAB expression patterns. Frozen cryosections would be a useful material for locating the endogenous GFP fluorescence and will be carried out in future studies.
However, there were some interesting areas of expression which did appear to be true and strong staining. At the e15.5 stage only, Wt1 expression was seen in the cells lining the inner surface of the bone, between the bone cells and hypertrophic chondrocytes. This was observed under DAB immunohistochemistry (Figure 6.6) and immunofluorescence staining (Figure 6.7). The stains were very strong and specific suggesting this to be true expression. Some interdigital staining was also observed for GFP and Wt1 at e14.5 (GFP only), e15.5, and e16.5, but as discussed earlier there was a large amount of auto-fluorescence and it was difficult to determine whether this was true staining.

The loss of Wt1 expression had no obvious effect on the bone morphology when studied at e18.5, but the preliminary data did suggest a reduction in non-haematopoietic triple+ MSC cells however this needs to be verified. The apparent increase in non-haematopoietic cells seen following the deletion (Figure 6.11) is more likely due to a decreased haematopoietic population. This would be due to reduced fetal liver haematopoiesis and it would therefore be interesting to assess Wt1 expression in the fetal liver. Loss of Wt1 expression also seems to have little effect on the percentage of hindlimb cells positive for osteoblast progenitor markers. These cells make up the majority of the hindlimb cells and throughout development may be changing in their adipogenic capabilities. This would not be too surprising as it is known that adult marrow stromal cells have a certain amount of plasticity. This is demonstrated by adipocytes being capable of osteogenesis in vivo (Bennett et al., 1991) as well as adipocytes differentiating from pericytes to fill any inactive empty areas of the marrow cavity with fat (Bianco et al., 1988; Bianco et al., 2001). Although it is not affecting the osteoblast progenitor populations, Wt1 loss may possibly affect the number of immature MSCs characterised as Sca-1+CD166+. These preliminary data only comment on the percentage of cells expressing specific markers however, and not their functional capabilities, such as differentiation. These markers were also characterised using adult cells and therefore may not relate directly to these embryonic cells.

The lineage tracing model, induced in utero at e14.5, showed that long thin cells positive for GFP and negative for Wt1 were found running along the edge of the
bone. The fact that these cells are positive for GFP and negative for \textit{Wt1} means that they have originated from a \textit{Wt1} expressing cell. These cells were observed at e18.5 and day 10, but not at 3 weeks of age. Flow cytometry of the 3 week bone and marrow samples confirmed the lack of GFP positive cells. These GFP$^+$ cells, with their long thin processes located in close proximity to the bone surface, are similar to the cells expressing \textit{Wt1} at e15.5 (Figure 6.6 and 6.7) and could potentially be derivatives of these cells. The fact that GFP$^+$ cells are seen at e15.5 supports the lineage tracing model induced at e14.5, and also may explain why it was difficult to identify Wt1 expressing cells in the embryonic limb prior to this stage. These data provide some evidence for a layer of \textit{Wt1} expressing cells lining the bone surface during development, although the GFP$^+$ lineage tracing cells appear nearer the periosteal bone surface (outer) and the \textit{Wt1} positive e15.5 cells seem to be in the endosteal region (inner bone surface). These locations are interesting as MSCs have been located near the endosteum of the bone, where they interact with bone lining osteoblasts (Muguruma \textit{et al.}, 2006) and the periosteal region also contains cells and MSCs which are capable of chondrogenesis (O'Driscoll and Salter, 1984). Therefore it is important to fully identify these GFP$^+$ and \textit{Wt1}$^+$ cells and their specific location.

This lineage tracing data are initially encouraging but do question why there are not greater numbers of GFP positive cells. Having seen GFP-positive \textit{Wt1}-negative cells at e18.5 you would expect the number of GFP positive cells to increase by day 10 and 3 weeks as the cells proliferate and their GFP$^+$ progeny are carried forward. There are several points of investigation for this, such as the GFP$^+$ cells being short term and therefore only a few are observed at any one time until none are seen by 3 weeks of age. Another alternative issue may be due to the lineage tracing construct itself such as inefficient tamoxifen administration and CreER activation. It would also be interesting to experiment with time of induction as there may be a particular time point when \textit{Wt1} is expressed highest during development and if induction is occurring too late these cells will be missed. Finally, \textit{in vitro} culture of these cells, which is currently underway, will be useful for following these cells along differentiation pathways.
In conclusion, this chapter shows *Wt1*-GFP$^+$ cells located for the first time in the developing hindlimbs. It also showed that at e15.5 these Wt1$^+$ cells are located at the endosteum. Lineage tracing showed that when induced at e14.5 and analysed at e18.5 and 10 days of age GFP$^+$ cells were located at the periosteum. Although a lack of phenotype was seen following *Wt1* deletion this may not be a true reflection as often phenotypes are not observed in the legs and bone until the mice reach weight-bearing age. As well as taking this into account, it would also be worth checking other bone sites, such as vertebrate and skull, in future work.
Chapter 7

Discussion and Future Work
7.1. Introduction

The aims of this project were to determine the role of Wt1 in the bone marrow with regard to mesenchymal stem / progenitor cell lineages; to characterise the Wt1 expressing population of cells and to determine the role of Wt1 in the maintenance and / or differentiation of these cells.

Throughout this thesis I have amassed evidence to suggest that Wt1 does play a role in bone biology.

7.2. Wt1 expressing cells in the bone and marrow

Bone and marrow cells expressing Wt1 mRNA also express the GFP protein in Wt1-GFP mice, and the percentage of these cells increases dramatically when cultured in vitro under hypoxia. The non-haematopoietic MSC portion of bone and marrow cells (i.e. Lin⁻CD3¹CD105⁺CD29⁺CD73⁺) contains GFP positive cells showing that the Wt1⁺ population overlaps slightly with the MSC population (as defined by those three markers). These three MSC markers are by no means definitive and could be restricting the true MSC values somewhat. On the other hand, as the overlap is relatively small, the GFP population may be largely made up of progenitor cells rather than stem cells.

Deletion of Wt1 failed to produce any obvious effect on the distribution of marrow and bone cells into various sub-populations, including the non-haematopoietic MSCs. However, there was a reduction in the percentage of GFP cells positive for the 3 MSC markers following in vivo deletion in the marrow, despite a lack of effect seen in vitro.

These minimal effects in vivo are surprising due to the dramatic phenotype (i.e. bone and fat loss, plus reduction in osteoblast synthesis) seen following in vivo deletion in adult mouse organs by Chau et al. (2011). The effects seen in the adult knock out could be due to systemic non-autonomous factors changing, whereas this present study is solely testing for changes in the distribution of MSCs. It is also difficult to
know why deletion is having such a small effect in vitro apart from the fact that more time may be needed to see any effects, or as previously covered, it is having no effect on cell distribution, but may be affecting their functional ability resulting in the adult bone loss phenotype.

Preliminary assessment of the functional capabilities showed that in vitro Lin-CD31- Triple-GFP+ and GFP- cells appeared capable of tri-lineage differentiation; i.e. into adipocytes, chondrocytes, and osteoblasts. These results need validating with specific lineage staining to assess whether there is any difference between the GFP+ and GFP- negative cells, before looking at what effect deleting Wt1 has on differentiation.

During in vitro adipogenesis the GFP+ percentage of marrow cells increases, peaking at day 5, before decreasing once terminally differentiated by day 12. This trend was mirrored by Wt1 expression in the cells cultured under hypoxia. Together this suggests that Wt1 may be playing a role in the adipocyte progenitor population, but less so in the terminally differentiated fat cells. This complements recent work from the Hastie laboratory showing Wt1-GFP positive cells present in the fat progenitor population, but absent from the mature fat cell population (Chau et al., 2014).

7.2.1. The effect of Wt1 deletion on total cell genetic expression patterns

Wt1 deletion in the bone has a negative effect on the expression of genes associated with developmental processes, including development of mesoderm, cartilage, tooth mineralisation, blood vessels, and skeletal systems, all of which originate from mesenchymal cells. The loss of Wt1 expression in the bone also causes an increase in expression of genes associated with inflammatory processes and immune responses. As we have shown, Wt1+ cells make up a very small percentage of the total population, yet when it is deleted we see significant effects on genetic expression pathways, suggesting that some of these effects, at least, are paracrine. It may also be that Wt1 is actively expressed in all cells, as even the GFP- express low levels as was shown in Chapter 3; this can be determined by single cell sequencing.
All the GO terms found to be enriched in the total bone cell microarray were successfully validated by the qRT-PCR of associated genes.

As I discussed in Chapter 5, and will go on to talk about further in this chapter, CreER recombinase activation alone negatively affected colony forming abilities. Therefore CAGG-CreER\textsuperscript{T2};Wt1\textsuperscript{+/+} samples were included when validating differentially expressed genes to determine whether Cre activation was also having an effect at a transcriptional level. Although only one sample was available for this Cre effect control, any gene which appeared to be affected was discarded from the study and so only genes not affected by CreER activation are discussed here.

Various inflammatory and immune GO terms were increased in the CAGG-CreER;Wt1\textsuperscript{loxP/loxP} mutant cells and an increase in the expression of 9 genes involved in these processes was validated by qRT-PCR. I also showed that loss of Wt1 in the bone marrow causes a significant increase in Cxcl10 as well as Cxcl1, a chemokine which is involved in bone formation (Onan \textit{et al.}, 2009). There is already evidence that Wt1 regulates chemokines, including Cxcl10, in the developing heart and kidneys, both directly and indirectly through the transcription factor IRF7 (Velecela \textit{et al.}, 2013), however this is the first time it has been observed in the bone.

91 genes relating to various enriched GO terms were validated by qRT-PCR. Interestingly 2 genes related to cellular nitrogen metabolism pathways were strongly reduced following Wt1 deletion; Arg1 and Ddah1. DDAH1 regulates nitric oxide synthase (NOS) activity by degrading the NOS inhibitor ADMA (Pope \textit{et al.}, 2009). A reduction in DDAH1 inhibits nitric oxide synthesis and is associated with endothelial dysfunction (Pope \textit{et al.}, 2009). Arginine, which is a vital substrate necessary for nitric oxide synthesis (Palmer \textit{et al.}, 1988) is converted to urea by the Arg1 product Arginase; the last enzyme in the urea cycle (Iyer \textit{et al.}, 1998). Wt1 may, therefore, be regulating the urea cycle and nitrogen metabolism pathway, with the loss of Wt1 and disruption of these pathways resulting in cellular toxicity. This may explain why the adult Wt1 knock out phenotype is so severe in such a short amount of time.
and of these 21 are direct binding targets of Wt1, as shown by kidney ChiP-Seq data (Hartwig et al., 2010 and personal communication with Andréas Schedl and Abdel Essafi). These data were obtained from kidney samples so theoretically there could be kidney specific targets plus other Wt1 targets amongst my list which are specific to the bone cells. These direct targets of Wt1 are discussed further below:

**Wt1 targets involved in cartilage development**

*Col5a1, Prrx1, Prrx2, Zeb1, Thbs1,* and *Shox2* are all involved in cartilage development and are significantly reduced in the bone cells following *Wt1* deletion. These 6 genes are also *Wt1* binding targets, in the kidney at least.

*SHOX* mutations in humans are associated with short stature conditions and the paralogous gene *Shox2* in mice is required for the differentiation of MSCs into mature chondrocytes (Bobick and Cobb, 2012). It has been shown to act upstream of *Runx2*, which as discussed in the Introduction chapter is important for chondrogenesis (Cobb et al., 2006).

*Prrx1* and *Prrx2* genes are also vital for normal bone formation. Loss of *Prrx1* results in abnormal craniofacial, limb, and vertebral skeletogenesis due to defective formation of chondrocyte and osteoblast precursors (Martin et al., 1995). Loss of *Prrx2* alone results in no skeletal phenotype, but lost together with *Prrx1* in a *Prrx1/Prrx2* double mutant knockout several abnormalities are seen which differ from the single *Prrx1* knockout (ten Berge et al., 1998). These included lower jaw, inner ear, and limb abnormalities. The two genes are expressed in the perichondrium and may regulate crosstalk between the chondrocytes and perichondrium which controls chondrocyte proliferation in the limb bones (ten Berge et al., 1998).

The fact that these genes, which are so important for bone and cartilage formation, are direct targets of Wt1 goes some way to explaining the bone loss phenotype seen following the *in vivo* deletion. Future work will include carrying out ChIP-Seq on bone and marrow cells and working towards identifying pathways involved in these phenotypes.
Wt1 targets involved in angiogenesis

Thbs1, Foxs1, Ptgis, Sulfl, Vegfa, Sphk1, Mmp9, and Efna1 are all involved in angiogenesis and are significantly reduced in the bone cells following Wt1 deletion. These 8 genes are also Wt1 binding targets, in the kidney at least.

VEGFa is mainly involved in angiogenesis and neovascularisation, especially in hypoxic or ischemic tissues, and its expression almost mirrors Wt1 expression in the coronary vessels of infarcted hearts (Wagner et al., 2002). Bone formation during development is reliant on angiogenesis and marrow vascularisation (Hunter et al., 1991) but is, however, not involved in adult bone homeostasis. This suggests that this is a secondary phenotype of Wt1 deletion, which is not directly related to the drastic adult bone loss in vivo.

Wt1 targets involved in EMT and Wnt signalling

Zeb1, Sox4, Fgfr2, Tgfb3, Wtip, and Smad3 are genes involved in EMT and Wnt signalling and are significantly reduced in the bone cells following Wt1 deletion. These 6 genes are also Wt1 binding targets, in the kidney at least.

Zeb1 is involved in regulating EMT by repressing epithelial genes and activating mesenchymal genes, such as collagens (Liu et al., 2008). When overexpressed it causes EMT by acting as a transcriptional repressor of E-cadherin and interacting with Snail; during cancer this results in metastasis (Guaita et al., 2002). To add to this, Wtip is also involved in EMT regulation as it interacts as a corepressor with Snail proteins to downregulate E-cadherin (Langer et al., 2008). Snail1 was significantly down-regulated in the Wt1 mutant bone cells and is directly regulated by Wt1 in the epicardium (Martínez-Estrada et al., 2010) and in the kidney (Essafi, unpublished). Mutation of Zeb1 results in decreased mesenchymal and neural progenitor proliferation leading to abnormal development of craniofacial skeleton, limb skeleton, and the CNS, as well as resulting in mesenchymal to epithelial transitions (Takagi et al., 1998; Liu et al., 2008). TGF-β ligand binding and Smad3
phosphorylation are also vital for EMT during development, and TGF-β expression also drives it during cancer (Reviewed by Zavadil and Böttinger, 2005).

Wt1 has already been shown to regulate EMT and MET in the epicardium and kidney respectively, via Wnt4 chromatin switching (Essafi et al., 2011). However, EMT has only been observed during development; these novel data show the functional importance of Wt1 expression on genes involved in adult EMT. This is a new and exciting finding and could lead to better understanding the role of EMT in adult tissue homeostasis, and the pathways involved.

7.2.2. The effect of Wt1 deletion on non-haematopoietic GFP+ genetic expression patterns

Not only did Wt1 deletion affect the expression of genes in the total bone cells, but also in the non-haematopoietic GFP+ population. Loss of Wt1 expression in the GFP+ cells downregulated genes associated with lipid synthesis and metabolism, characteristic of adipocytes. In a preliminary experiment I showed that GFP+ cells are capable of adipocyte differentiation and Chau et al. (2014) showed that adipocyte progenitors express Wt1. Therefore the downregulation of lipid synthesis genes is not too surprising but offers new genes to explore the mechanisms involved.

Fewer genes were affected by Wt1 loss when the cells were sorted into GFP+ and GFP− populations. It is therefore possible that FACS is having a negative effect on transcription and the RNA quality of these cells.

7.2.3. The effect of Wt1 deletion on non-haematopoietic GFP− genetic expression patterns

These microarrays also allowed me to look at the GFP− population. When Wt1 was deleted genes downregulated were related to a mixture of GO terms, probably due to the heterogeneity of these cells. Ossification was one such term; Wt1 deletion is therefore down-regulating genes involved in bone formation in the cells which do not express Wt1, presumably through a paracrine signalling pathway.
7.2.4. The expression pattern differences between non-haematopoietic GFP\(^+\) and GFP\(^-\) cells

Genes upregulated in the GFP\(^+\) population compared to the GFP\(^-\) are associated with several sterol, lipid, and cholesterol terms. This corresponds with the reduction in these genes following \(Wt1\) deletion in the GFP\(^+\) cells (discussed above) and the role that \(Wt1\) evidently plays in adipogenesis.

Bone mineralisation genes are more highly expressed in the GFP\(^-\) population compared to GFP\(^+\). Again this adds to the evidence of paracrine interactions between the GFP\(^+\) and GFP\(^-\) populations, as these are the same genes which are reduced following \(Wt1\) deletion.

The fact that the GFP\(^+\) and GFP\(^-\) populations show little variation in gene expression suggests that \(Wt1\) expression may be cycling, or may be having a systemic paracrine effect on both GFP\(^+\) and GFP\(^-\) populations. Also, the qRT-PCR data showed that the GFP\(^-\) sorted cells still express \(Wt1\), just at around 4 times lower than GFP\(^+\), meaning that GFP\(^+\) sorted cells are purely enriched for the most highly expressing cells; this could again be due to cycling.

7.3. \(Wt1\) expression and hypoxia

Hypoxia caused a huge effect on the GFP percentage and as discussed in Chapter 3, 3\% Oxygen more closely mimics physiological conditions than standard culture conditions (i.e. 20\% Oxygen) (Fehr et al., 2007). However, I showed here that, with regard to GFP percentage, the \textit{in vivo} data are more closely matched to the \textit{in vitro} normoxic results rather than hypoxic. However, it should be noted that the \textit{in vitro} values are taken from an adherent population of cells which have been cultured for 7 days, making them less comparable with the \textit{in vivo} cells.

The increased percentage of GFP cells could be a result of hypoxia induced proliferation or hypoxia induced \(Wt1\) expression. Wagner \textit{et al.} (2003) have shown HIF-1 binds \(Wt1\) in the kidney and heart; identifying it in the bone and marrow would be novel and therefore an area of future interest and research.
Hypoxia related genes were significantly downregulated following the loss of *Wt1*. *Egln3* was one of the genes validating the hypoxia GO term enriched in the microarray. As discussed in Chapter 5, *Egln3* is involved in weakening HIF activity, with HIF driving *Egln3* transcription in a negative feedback loop (Pescador et al., 2005). *Wt1* deletion significantly reduces *Egln3* expression whilst HIF-1 activates *Wt1* (Wagner et al., 2003), meaning *Wt1* may also be adding to this negative feedback loop.

I was able to identify some of these down-regulated genes as *Wt1* targets using the kidney ChiP-Seq data mentioned previously (Hartwig et al., 2010 and personal communication with Andréas Schedl and Abdel Essafi).

*Wt1 targets involved in hypoxia*

*Fosl2, Mmp2, Vldlr, Wtip, Ptgis, Vegfa, Smad3, Tgfβ3, and Sox4* are genes involved in hypoxia and are significantly reduced in the bone cells following *Wt1* deletion. These 9 genes are also *Wt1* binding targets, in the kidney at least.

I have already mentioned that *Vegfa* is a target gene of HIF-1 (Semenza, 1996). Under hypoxic conditions, such as tumours, *Vegfa* as well as TGF-β are produced to attract macrophages and monocytes which support growth and angiogenesis (Reviewed by Majmundar et al., 2010). A study of prostate carcinomas showed that EMT was induced by hypoxia and TGF-β which causes a significant decrease in estrogen receptor β expression, and increase in VEGFa expression. This loss of ERβ promoted EMT and increased the VEGFa-mediated translocation of Snail1, another target of *Wt1*, from the cytoplasm to the nucleus (Mark et al., 2010). As well as VEGFa, Sox4 was identified as a hypoxia target in cancer (Lendahl et al., 2009); as both are also targets of *Wt1*, and *Wt1* is a target of HIF-1 (Wagner et al., 2003) there is evidently a complex mechanistic pathway to be identified. This shows how interlinked many of these mechanisms are; several hypoxia related *Wt1* targets overlap with the *Wt1* targets involved in EMT pathways.
The microarray data indicated, unsurprisingly, responses to oxygen levels and hypoxia GO terms in the hypoxic cells compared to normoxic, but glycolysis was the most highly enriched. Glycolysis is used as an adaptive strategy by tumour cells for the survival of hypoxic cells within the tumour and for the growth of normoxic cancer cells (Guillaumond et al., 2013). Glycolysis in tumours is usually associated with tumour survival and aggressiveness (Guillaumond et al., 2013) which fits nicely with Wt1's role as a tumour suppressor during development.

Genes involved with bone remodelling and bone resorption were higher in the normoxic cells rather than hypoxic. The hypoxic nature of the bone marrow stem cell niche is vital for maintaining the quiescence of stem cells. In increased oxygen levels, found in close proximity to the bone and its vasculature, cells lose their quiescence and are more likely to differentiate (Fehrer et al., 2007; Mohyeldin et al., 2010). Therefore, it is not unexpected that under hypoxia bone remodelling is reduced as this is when the cells are most quiescent.

7.4. **Wt1 expressing adult osteoblast progenitors**

CD166\(^{-}\)Sca-1\(^{-}\) and CD166\(^{+}\)Sca-1\(^{-}\) populations were enriched for osteoblasts and had osteogenic potential when characterised by Nakamura et al. (2010). I showed that in vivo over 70% of the GFP population were found to be in these two osteoblast progenitor populations in the marrow, and over 80% in the bone.

Deletion of *Wt1* in vivo did not affect the proportion of GFP cells in these populations, but it did drastically reduce the percentage of the total osteoblast progenitor cells which were GFP positive. Further work is needed to assess whether the function of these cells was affected at all, e.g. ability to differentiate, as was seen by Chau et al. (2011).

On the other hand, *in vitro* GFP\(^{+}\) bone and marrow cells are mainly found in the CD166\(^{+}\)Sca-1\(^{-}\) population which Nakamura et al. (2010) states as an uncharacterised population. Therefore, culturing these cells seems to affect their expression of particular cell surface markers and their osteogenic capabilities.
7.5. The effect of activated CreER recombinase

The CFU-F assay to determine what effect \( Wt1 \) deletion had on colony formation produced such dramatic results that we investigated what effect the activated CreER recombinase alone was having. It was the magnitude of the effect that first aroused suspicions; if \( Wt1 \) is expressed in such a small percentage of cells then how is it causing such a great effect? The same significant reduction in colony number was observed in the activated CreER recombinase samples showing that \( Wt1 \) expression was irrelevant. Cells positive for CreER recombinase were activated by the addition of tamoxifen and compared with controls; cells positive for CreER recombinase plus ethanol, and cells negative for CreER recombinase. Very few studies control for CreER toxicity effects. This raises questions as to whether activated CreER recombinase is the cause of some phenotypes seen in published studies also using this construct. The initial ubiquitous deletion of \( Wt1 \) by Chau et al. (2011) was controlled for using CreER recombinase positive mice with no \( loxP \) sites (CAGG-CreER\textsuperscript{TM} positive; \( Wt1^+/+ \)). There were no significant effects on tissue morphology as observed by pathologists following CreER activation \textit{in vivo}, however \textit{in vitro} effects including CFU-F assays were not checked (Chau et al., 2011). The lack of morphological effects is possibly because the body is better at coping with Cre toxicity by removing it from the cells or compensating for its effects, unlike \textit{in vitro} cells which are cultured in wells and unable to escape the activated CreER recombinase.

However, when transcriptomics were carried out on these cells only 15 genes changed significantly, and even then the log fold change was under 1.5. When the entire data set compared expression changes as a whole 4 GO terms of interest were enriched in the activated CreER cells (i.e. Cre\textsuperscript{+}). These were bone resorption, regulation of bone resorption, regulation of bone remodelling, and regulation of bone mineralisation. Although 20 genes were up-regulated in these GO terms none of them were significantly so. CAGG-CreER\textsuperscript{T2} positive; \( Wt1^+/+ \) samples were not seen as outliers when normalised using principal component analysis and a cluster dendrogram, which also suggests that activated CreER is not having a significant effect on gene expression. However, it is interesting that bone resorption and
increased osteoclasts were seen when \( Wt1 \) was deleted \textit{in vivo} (Chau \textit{et al.}, 2011), making it something worth investigating further and validating with qRT-PCRs. Although, the results from this microarray are carried out on bone cells cultured \textit{in vitro} for 7 days and are therefore not 100% comparable to results found \textit{in vivo}.

As described earlier, when CAGG-CreER\textsuperscript{T2} positive;\( Wt1^{+/+} \) samples were included as controls in qRT-PCR validations only some genes were affected by activated CreER recombinase. Interestingly, most of these were inflammatory genes. This inflammatory aspect complements the inability to form colonies, possibly due to increased cell death.

There is no doubt that activation of CreER recombinase is negatively affecting the ability of these cells to form colonies. It is possible that the CreER recombinase enzyme is cleaving the DNA at endogenous pseudo \textit{lox} sites which occur naturally in the genome and share some homology with \textit{loxP} sites (Thyagarajan \textit{et al.}, 2000). This toxicity of Cre has been previously shown in mammalian cells (Loonstra \textit{et al.}, 2001; Pfeifer \textit{et al.}, 2001; Silver and Livingston, 2001).

Cells expressing Cre recombinase and lacking \textit{loxP} sites undergo cell cycle arrest at the G2/M phase inhibiting cell growth (Pfeifer \textit{et al.}, 2001) and have chromosomal aberrations which lead to genetic instability (Loonstra \textit{et al.}, 2001; Silver and Livingston, 2001). The use of Cre in the generation of knockin animals means that phenotypes associated with the knockin allele may also be due, in part, to Cre-mediated mutations (Silver and Livingston, 2001).

Cells with the CreER knockin allele driven by the endogenous ROSA26 promoter, similar to the CAGG driven CreER used in this study were investigated for toxicity effects by Loonstra \textit{et al.} (2001). When these cells, which lack \textit{loxP} sites, were cultured with tamoxifen a severe reduction in proliferation was also observed (Loonstra \textit{et al.}, 2001). This data corroborates with the lack of colony growth seen by my CAGG-CreER positive cells and confirms that I am seeing a toxic effect.

In Chapter 3, the deletion of \( Wt1 \) has an effect on the non-haematopoietic MSC percentage of the GFP population, but has no effect on the other populations. If this were due to Cre toxicity then the other GFP and total populations should also be
affected. Therefore, this result must be due to *Wt1* deletion, even if Cre is having a toxic effect and potentially masking other *Wt1* deletion effects.

7.6. A sex effect on colony forming abilities

In the females only, the CreER recombinase negative (and therefore control) mice showed significantly higher colony numbers when cultured with tamoxifen rather than EtOH. There are links between tamoxifen, the estrogen receptor, and bone growth which make this gender dimorphism an interesting observation.

There are various gender differences found within stem cell groups, including MSCs (reviewed by Ray *et al.*, 2008). Within the MSC population, hypoxia-induced VEGF is increased in female MSCs compared to male (Crisostomo *et al.*, 2006). The qRT-PCRs in this study were carried out using male bone cell samples. *Vegfa*, which is a target of Wt1, was shown to be significantly downregulated following *Wt1* deletion under hypoxia. It would be interesting to see whether this response is also seen in the female bone samples. The osteogenic properties of MSCs are also greater in cells cultured with 17β-estradiol, which show an increase in BMP, osteocalcin, calcium deposit, as well as *ALP*, *Collagen I*, and *TGFβ1* gene expression (reviewed by Ray *et al.*, 2008). These genes were also affected by *Wt1* in the microarray, carried out on male samples. Again, it would be interesting to compare with female samples.

Tamoxifen has been shown to have estrogen agonistic effects on human bone tissues with bone cell numbers found to increase, as well as the number of cells in the S phase, when treated with tamoxifen (Miki *et al.*, 2009). Tamoxifen was also found to inhibit bone resorption and osteoclast formation in an estrogen receptor dependent manner (Michael *et al.*, 2007).

It is therefore unsurprising that the female bone cells with increased osteogenic properties combined with the estrogen agonistic effects of tamoxifen produce higher colony numbers when cultured with tamoxifen than with EtOH.
7.7. The effect of inactivated CreER recombinase

As well as activated CreER recombinase having an effect as discussed above, it seems that the inactivated state is also having an effect. Cells positive for CreER recombinase formed significantly more colonies than cells which were negative for the CreER recombinase. These cells were cultured with ethanol, therefore CreER recombinase was not activated, suggesting that the mere presence of the CreER construct is the only difference between these cells.

As the CreER construct seemed to be having an effect alone, I next checked whether the \textit{loxP} sites could be affecting the CFU-F assays. The presence of \textit{loxP} sites had no effect on the colony forming abilities of male cells, but did seem to have a positive effect on the number of female colonies. As the same sex-effect is being seen by cells cultured with tamoxifen it needs to be determined whether it is actually one, the other, or both together. It would also be worthwhile to consider possible background effects as WER (CAGG-CreERT\textsubscript{2};\textit{Wt1}\textsuperscript{loxP/loxP}) mice have mixed background compared to the pure background of the CAGG-CreERT\textsubscript{2} negative;\textit{Wt1}\textsuperscript{+/-} mice.

It has been shown that CreERT\textsubscript{2} can be leaky meaning that it can still be exerting an effect in the nucleus without the presence of a 4-OH tamoxifen ligand (discussed by Vooijs et al., 2001) as well as spontaneous \textit{loxP} site recombination due to tamoxifen contamination between animals \textit{in vivo} (Brake et al., 2004). However, as shown in Section 7.5 active CreERT\textsubscript{2} results in a lack of colonies compared to CreERT\textsubscript{2} negative cells, where as here when CreERT\textsubscript{2} is cultured with ethanol there is a significant increase in colonies (again compared to CreERT\textsubscript{2} negative cells). This suggests that spontaneous Cre activation is not occurring within this time frame, but may occur over long term culture therefore it will be considered when following up these various Cre phenotypes.
7.8. Tracing the lineage of *Wt1* expressing cells in adults

Technical difficulties were encountered during adult lineage tracing experiments. No GFP cells were observed when the lineage tracing model was induced during adulthood despite the fact that *Wt1* is endogenously expressed during this time. In Chapter 4 I discussed whether this problem was due to possible timing and / or dosing issues. Although this does not explain why GFP is not being expressed even at endogenous *Wt1* levels it may still be a factor.

Therefore lineage tracing was carried out earlier with induction occurring *in utero* (discussed later in this chapter).

7.9. *Wt1* expression during development

The preliminary data from this thesis suggest that *Wt1* plays an important role during limb development, along with its importance for various other organs (Kreidberg *et al.*, 1993). GFP\(^+\) cells are present in the developing limb from e11.5 to e16.5 but decrease across this time span. *Wt1* expression may be more important for early development, perhaps for the differentiation of progenitors into osteoblasts and chondrocytes. Interestingly, *Wt1* mRNA levels also decrease from e11.5 to e16.5 in epicardial cells during heart development (Velecela *et al.*, 2013). Velecela *et al.* (2013) hypothesize that the decline in *Wt1* expression coincides with the maturation and quiescence of epicardial cells and is involved in inhibiting the embryonic program. This may also be the case for limb development.

Despite the flow cytometry results showing GFP\(^+\) cells present during limb development, there was very little immunohistological staining to confirm this. GFP and Wt1 positive mesothelial cells were observed at e11.5, e14.5, and e16.5, which acted as a positive control (Armstrong *et al.*, 1993; Walker *et al.*, 1994; Wilm *et al.*, 2005; Que *et al.*, 2008; Chau and Hastie, 2012; Cano *et al.*, 2013).

However, at the e15.5 stage only, Wt1 expression was seen in the cells lining the inner surface of the bone (endosteal region), between the bone and hypertrophic chondrocytes which will become the marrow cavity. At e18.5 and 10 days the
lineage tracing model showed long thin GFP\(^{+}\)Wt1\(^{-}\) cells running along the outer edge of the bone (periosteal region). These cells originated from Wt1\(^{+}\) cells and have a similar morphology to the endogenous Wt1\(^{+}\) cells seen at e15.5. This suggests that they may be the same cells which could be progenitors forming the bone or migrating from one side to the other. MSCs have been located near the endosteum of the bone, where they interact with bone lining osteoblasts (Muguruma et al., 2006) and MSCs capable of chondrogenesis are located at the periosteal region (O’Driscoll and Salter, 1984).

Some weak interdigital GFP and Wt1 staining was also observed at e14.5 (GFP only), e15.5, and e16.5. A study using a Wt1 promoter driven β-galactosidase reporter also showed interdigital mesenchyme expression during limb development (e12.5 – e15.5) suggesting that Wt1 may play a role in interdigital apoptosis plus limiting the digital chondrogenesis (Moore et al., 1998). Wt1 is known to regulate retinoic acid signalling via Raldh2 (Guadix et al., 2011), and retinoic acid induces interdigital apoptotic pathways (Dupé et al., 1999) making this an interesting pathway to investigate.

Osteoblast progenitors make up the majority of the hindlimb cells and these preliminary data suggest that throughout development their adipogenic capabilities are changing. Marrow stromal cells have a certain amount of plasticity demonstrated by adipocytes being capable of osteogenesis in vivo (Bennett et al., 1991) as well as adipocytes differentiating from pericytes to fill any inactive empty areas of the marrow cavity with fat (Bianco et al., 1988; Bianco et al., 2001). Future work will be carried out to verify the preliminary data on Wt1\(^{+}\) effect on mesenchymal cells and osteoblast progenitors during development. As of yet there is not enough data to form any conclusions. The effect on chondrocyte and adipocyte progenitors will also be investigated.

This lineage tracing data is initially encouraging but does provide several areas for future work. The presence of GFP\(^{+}\)Wt1\(^{-}\) cells at e18.5 would lead you to assume an increase in GFP\(^{+}\) cells by day 10 and 3 weeks due to proliferation and continuation of the GFP\(^{+}\) line, but this does not seem to be the case. The GFP\(^{+}\) cells may be short term and therefore only a few are observed at any one time until none are seen by 3
weeks of age. Another alternative issue may be due to the lineage tracing construct itself such as inefficient tamoxifen administration and CreER activation. Finally, it would also be interesting to experiment with the time of induction as there may be a particular time point when $Wt1$ is expressed highest during development and if induction is occurring too late these cells will be missed. These are all avenues down which this work can be taken forward.

7.10. Future Work

I have already mentioned several experimental areas for future work in the discussion above. It is important to ascertain whether loss of $Wt1$ is having any functional effect on the Triple$^+$ MSC cells and osteoblast progenitors. This would include assessing the proliferation and differentiation capabilities of both during adulthood and development. Proliferation assays will also be carried out on the GFP$^+$ population cultured under hypoxia and normoxia to determine whether proliferation is the cause of the increased GFP percentage.

When observing the osteoblast progenitor population a proportion of GFP$^+$ cells were found to be CD166$^+$Sca-1$^-$. This population is as yet uncharacterised and as some of my cells of interest fall into this category it is important that I understand what they are.

There have been a couple of technical difficulties with the lineage tracing model, at both adult and developmental stages, although at developmental stages it worked significantly better. Currently there is concern that the $Wt1$-CreER$^{T2}$ construct might not be expressed in all the endogenously expressing sites, for example in the stellate cells (Personal communication with Tim Kendall and Andréas Schedl). Therefore, it may be worth further investigation into the marrow and bone cells using the constitutively active $Wt1$-Cre model as described in Chapter 4 (Personal communication between the Hastie laboratory and Ramón Muños-Chápuli). Optimisational experiments will also be carried out to investigate the effect of induction time and tamoxifen dosage.
ChIP-Seq will be carried out on bone and marrow cells in order to identify and confirm \textit{Wt1} targets in this cell set. Using these data I will then look for enhancers and \textit{Wt1} binding elements in order to identify pathways involved in the bone and cartilage phenotypes. The ChIP-Seq data will also be useful in combination with the qRT-PCR results from this study to investigate various pathways and mechanisms, such as the potential \textit{Egln} feedback loop, the intricate role \textit{Wt1} evidently plays with hypoxia induced genes, and \textit{Wt1} in adult EMT.

The final aspect of future work will be to study the effects of CreER recombinase in greater detail. The findings from this project concur with previous findings of toxicity with regard to cell growth and in this case colony growth. Colony formation was also affected by inactive Cre (i.e. cultured with EtOH), tamoxifen alone, and cell gender. These are all interesting areas to be investigating which could prove of great importance, not just to this study, but to future experimental procedures.

7.11. Conclusions

This thesis has uncovered several interesting findings. For the first time this shows that \textit{Wt1}+ cells are present in adult and developing bone and marrow. This population appears to contain possible MSCs, osteoblast progenitors, and are potentially capable of tri-lineage differentiation. During development these \textit{Wt1}+ cells are located in the periosteal region; an area known to contain cells and MSCs capable of chondrogenesis (O'Driscoll and Salter, 1984). Microarray analysis revealed that \textit{Wt1} expression is important for various pathways including bone, cartilage, fat, and skeletal development and angiogenesis. \textit{Wt1} deletion also affected genes indicativve of EMT. EMT has only ever been observed during development making this a very exciting finding and avenue of study.

Interdigital \textit{Wt1} expression was also observed during embryogenesis corroborating with similar staining seen by Moore \textit{et al.} (1998) and suggesting that Wt1 may play a role in interdigital apoptosis.
The data in this thesis also raises several questions regarding experimental conditions, particularly for CFU-F assays. CreER\textsuperscript{T2}, \textit{loxP} sites, tamoxifen, oxygen tension levels, and gender all exert specific effects on colony formation. Each variant will be followed up to determine the mechanisms behind these results.

In closing, the work from this project raises many new questions to be answered and shows \textit{Wt1} to be present and functionally important for the mesenchymal biology of bone and marrow.
References


Appendix
Appendix 1 shows a schematic diagram outlining the experimental strategy for the CFU-F assay.

```
Bone and Marrow harvested from CAGG-CreERTm, Wt1cre/+; p53D mice

↓

DAY 1: Plated at low density (0.5x10^6 cells/well) and high density (1x10^6 cells/well) in single cell suspension

↓ 3 days

DAY 3: PBS wash and fresh MesenCult® media with 4-OH Tamoxifen

↓ 3 days

DAY 6: Fresh MesenCult® (minus 4-OH Tamoxifen) every 2-3 days

↓ 10 days

DAY 16: PBS wash, stain with 0.5% Cresyl Violet Acetate and dry
```
Singlets were selected for and doublets excluded by plotting the Forward Scatter - Height (FSC-H) values against FSC-area (A). Singlets will have the same (or similar) values and therefore can be selected in the diagonal display (45 degrees, passing through zero). A representative image is shown in Figure A. Dead cells and debris were then removed by selecting the central population in a SSC-A vs. FSC-A plot (Figure B). Gating was carried out using a GFP negative and non-stained control. Representative flow cytometry plots are shown for GFP (Figure C&D), Lineage and CD31 (Figure E), CD73 (Figure F), CD29 (Figure G&H), CD105 (Figure I&J), Sca-1 (Figure K), and CD166 (Figure L).
E

F

G

H

I

J

K

L

226
Appendix 3

Osteoblast progenitor quadrants were established using GFP negative control mice. Representative flow cytometry plots are shown for marrow (Figure A) and bone (Figure B) cultured under normoxia, and marrow (Figure C) and bone (Figure D) cultured under hypoxia.
Appendix 4 shows a schematic diagram outlining the experimental strategy used for the total cell microarrays.

Appendix 5 shows a schematic diagram outlining the experimental strategy used for the Lin-CD31\(^+\)GFP sorted microarrays.
Representative flow cytometry plots for *in vivo* lineage tracing to highlight the lack of GFP$^+$ cells found in the marrow and bone after 4 doses and 1 day between final dosing and harvest. Figure A shows flow cytometry plots for GFP negative, Cre negative, and Cre positive bone marrow samples. Population 3 (P3) highlights auto-fluorescence allowing the GFP population to be gated for using the P4 gate. For bone marrow (Figure A) and bone (Figure B) there are no GFP positive cells present in the Cre positive samples, but 0.1% appear positive in the Cre positive spleen sample (Figure C).