Acute Multiple Organ Failure in Adult Mice Deleted for the Developmental Regulator Wt1

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Abstract

There is much interest in the mechanisms that regulate adult tissue homeostasis and their relationship to processes governing foetal development. Mice deleted for the Wilms’ tumour gene, Wt1, lack kidneys, gonads, and spleen and die at mid-gestation due to defective coronary vasculature. Wt1 is vital for maintaining the mesenchymal–epithelial balance in these tissues and is required for the epithelial-to-mesenchyme transition (EMT) that generates coronary vascular progenitors. Although Wt1 is only expressed in rare cell populations in adults including glomerular podocytes, 1% of bone marrow cells, and mesothelium, we hypothesised that this might be important for homeostasis of adult tissues; hence, we deleted the gene ubiquitously in young and adult mice. Within just a few days, the mice suffered glomerulosclerosis, atrophy of the exocrine pancreas and spleen, severe reduction in bone and fat, and failure of erythropoiesis. FACS and culture experiments showed that Wt1 has an intrinsic role in both haematopoietic and mesenchymal stem cell lineages and suggest that defects within these contribute to the phenotypes we observe. We propose that glomerulosclerosis arises in part through down regulation of nephrin, a known Wt1 target gene. Protein profiling in mutant serum showed that there was no systemic inflammatory or nutritional response in the mutant mice. However, there was a dramatic reduction in circulating IGF-1 levels, which is likely to contribute to the bone and fat phenotypes. The reduction of IGF-1 did not result from a decrease in circulating GH, and there is no apparent pathology of the pituitary and adrenal glands. These findings 1) suggest that Wt1 is a major regulator of the homeostasis of some adult tissues, through both local and systemic actions; 2) highlight the differences between foetal and adult tissue regulation; 3) point to the importance of adult mesenchyme in tissue turnover.


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Introduction

Although much is known about the mechanisms that govern cellular differentiation during development, we know less about the processes that regulate cell turnover and homeostasis in the adult. Perhaps the exceptions to this rule are rapidly turning over tissues such as intestine, skin and haematopoietic tissue. Recently it has been shown that genes required for regulating differentiation during foetal development may not be used in regulating turnover of the same tissues in the adult [1,2].

Mutation of the Wilms tumour gene, WT1, in humans may lead to the eponymous paediatric kidney cancer, glomerulosclerosis of the kidney and gonadal dysgenesis, which can manifest as male to female sex reversal [3]. During foetal development, Wt1 is expressed in the kidney, gonads, spleen, the mesothelium which surrounds most organs as well as ill-defined body mesenchyme. Knockout mice lack kidneys, gonads, and spleen and die at mid-gestation through the lack of coronary vasculature formation [4]. There are no apparent defects of the skeletal, haematopoietic, digestive, or metabolic systems.

Recently we have shown that Wt1 is a key regulator of the balance between the epithelial and mesenchymal states in a number of developing organs. Whereas it is required for the mesenchymal to epithelial transition (MET) underlying the formation of kidney nephrons, in the heart it is essential for the reverse process, the epithelial to mesenchyme transition (EMT) required for the production of proliferating cardiovascular progenitors from the epicardium (a mesothelium) [5]. In a similar vein Wt1 expressing mesothelial cells in the intestine and lung produce mesenchymal progenitors for vascular smooth muscle [6,7]. Furthermore, very recent evidence proves that, in the developing liver, Wt1 expressing mesothelial cells provide the
precursors for stellate cells [8,9,10]. Stellate cells in the liver and the pancreas have aroused much interest through their ability to regulate tissue fibrosis, via the production of cytokines [11,12].

The pancreas have been shown to be a source of mesothelial progenitors for stellate cells [8,9,10]. Stellate cells in the liver and pancreas have been shown to be important in maintaining the health of these organs. Our studies show that the control of adult tissue turnover may be different from that during foetal development. Further experiments showed that the tissue failure we observed is due both to local defects of stem/progenitor cell activities and to significant changes in the serum levels of some key master regulators. In particular there is a dramatic reduction in the levels of IGF-1, a key regulator of homeostasis and aging. Our studies also show that the control of adult tissue turnover may be different from that during foetal development. These findings have important implications for understanding and treating common human diseases.

**Results**

**Tamoxifen-mediated deletion of Wt1**

To enable inducible deletion of Wt1 in the adult, we generated tamoxifen inducible Wt1 KOs by crossing CAGG promoter driven Cre-ERTM mice with our homozygous Wt1 conditional mice, where the first exon of Wt1 is flanked by loxP sites [5]. Successful Wt1 deletion was demonstrated by recombination PCR and the depletion of Wt1 expression in mesothelia (Figure S1 and Figure S2). Deletion of Wt1 in the mesothelium did not affect the integrity of the tissue (Figure S3). The health status of the mutant animals deteriorated quickly and all the mice had to be culled by 10 days post-induction (p.i.). Prior to death, the mutant mice presented dramatic phenotypes; they were less active and oedemic. Upon dissection, fluid was sometimes found in the abdominal cavity and in the subcutaneous tissues. Detailed gravimetric analysis showed that there was a reduction in the spleen to body weight ratio as well as in the heart to body weight ratio (Table 1). Subsequent histological analysis revealed pale kidneys, severe spleen and pancreas atrophy, and deficiency of fat tissues. For most tissues, mice treated at 3, 10, or 13 weeks of age developed the same phenotypes. The only exception to this involved fat, as we discuss in more detail later. Before considering each phenotype, it is important to emphasise that not all tissues showed overt signs of damage. For example, we observed no obvious macroscopic changes to the lung, liver or intestine- three tissues often involved in systemic inflammatory responses. Furthermore, although there was a 30% reduction in the heart/body weight ratio there was no obvious cardiovascular pathology (Table 1).

**Table 1. Summary of the gravimetries of adult mice deleted for Wt1.**

<table>
<thead>
<tr>
<th>Mature mice</th>
<th>Weight</th>
<th>Spleen/BW %</th>
<th>Kidney/BW %</th>
<th>Heart/BW %</th>
<th>Liver/BW %</th>
<th>Testes/BW %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant</td>
<td>20.07</td>
<td>0.260±0.026</td>
<td>1.673±0.085</td>
<td>0.509±0.027</td>
<td>7.103±0.55</td>
<td>0.640±0.60</td>
</tr>
<tr>
<td></td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
<td>n=6</td>
</tr>
<tr>
<td>Control</td>
<td>20.87</td>
<td>0.586±0.033</td>
<td>1.548±0.053</td>
<td>0.714±0.093</td>
<td>6.780±0.34</td>
<td>0.793±0.053</td>
</tr>
<tr>
<td></td>
<td>n=12</td>
<td>n=9</td>
<td>n=10</td>
<td>n=11</td>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.563</td>
<td>0.000⁷</td>
<td>0.239</td>
<td>0.003⁷</td>
<td>0.849</td>
<td>0.073</td>
</tr>
</tbody>
</table>

**Author Summary**

It is important to understand the cellular and molecular pathways that regulate the maintenance and turnover of adult tissues. These processes often go awry in diseases and are likely to deteriorate with ageing. Here we show that removal of a single gene, the Wilms' Tumour gene, Wt1, in the adult mouse leads to the extremely rapid deterioration of multiple tissues. Within 7–9 days after gene removal kidneys fail, the pancreas and spleen suffer severe atrophy, there is widespread loss of bone and body fat, and red blood cells are no longer produced. Our findings reveal the vulnerability of adult tissues, while opening up avenues for dissecting the pathways controlling tissue turnover. Further experiments showed that the tissue failure we observed is due both to local defects of stem/progenitor cell activities and to significant changes in the serum levels of some key master regulators. In particular there is a dramatic reduction in the levels of IGF-1, a key regulator of homeostasis and aging. Our studies also show that the control of adult tissue turnover may be different from that during foetal development. These findings have important implications for understanding and treating common human diseases.

Thirdly, **Wt1** is mutated or overexpressed in acute myeloid leukaemia (AML) [20]. However, **Wt1** is not required for foetal haematopoiesis [21]. Given **Wt1** expression in adult bone marrow and association with leukaemia, we surmised that **Wt1** might play a role in adult haematopoiesis.

Finally, **Wt1** is expressed at high levels in most adult cancers studied [22], though expression has not been detected in the normal tissue counterparts. It has been proposed that **Wt1** might be an oncogene in adult cancer in contrast to its function as a tumour suppressor in paediatric kidney cancer [3]. As a prelude to testing this, it was necessary first to determine whether the gene is essential for normal development or maintenance of the epithelia from which these tumours arise.

To address these propositions, we deleted the **Wt1** gene ubiquitously in adult mice. While our findings inform on these issues, the results far exceed our expectations. The range, severity, and rapidity of the phenotypes observed were dramatic and unexpected and raise major questions about adult tissue homeostasis.
Deletion of Wt1 leads to acute glomerulosclerosis

Wt1 is crucial for kidney development as the conventional Wt1-null embryos suffer from renal agenesis [4]. Upon induction of Wt1 deletion in our model, expression of Wt1 in the podocytes was completely depleted (Figure 1B) and the mutant mice were shown to have severe proteinuria (Table 2). H&E staining showed that the tubules were filled with protein casts (Figure 1A, arrow). The mutant kidneys had well developed glomerulopathy with cytopathic changes in podocytes and parietal epithelium. There was almost complete loss of synaptopodin and nephrin expression in the podocytes in the mutant kidneys (Figure 1C and 1D). EM studies showed that the foot processes of the podocytes were completely lost in the mutant kidneys (Figure 1E, day 10 post-injection). The development of the kidney phenotype in our model was extremely rapid. Five days post-tamoxifen injection, H&E stained kidney sections showed normal histology while podocyte effacement started to appear (Figure 1F). At day 7 post-injection, protein casts in the tubules were already present and the glomeruli started showing signs of degeneration (Figure S4a). Finally, plasma levels of urea and creatinine were normal at day 5 p.i., started to rise at day 7 p.i., and were significantly elevated at day 10 p.i (Table 2). In our model, mice that were heterozygous for the Wt1 conditional allele (CAGG-CreERT², Wt1tm1-p/+tm1) did not exhibit any kidney abnormalities after tamoxifen-mediated deletion of Wt1. In addition, tamoxifen treated mice that were only positive for the CAGG-CreERT² allele and wild type for the Wt1 lost sites (i.e. CAGG-CreERT² positive; Wt1+/-) were also included as controls and did not demonstrate any phenotypes. The kidney phenotype in our model is similar to other nephrotic syndrome mice where Wt1 has been deleted specifically in adult podocytes. These describe below apart from the kidney defects. Most importantly, the development of the kidney phenotype in our model is similar to other nephrotic syndrome mice where Wt1 is crucial for kidney development as the conventional Wt1-null mice correlates with enhanced apoptosis in the primordial spleen cells [29]. In the adult Wt1 KO model, the mutant spleen was much paler and smaller in size compared with the control spleen (Figure 2A, arrow). There was a reduction in the number of proliferating cells in the mutant spleen; however the number of cells expressing an apoptotic marker (active caspase 3) remained unchanged (Figure S8A–S8D). The spleen to body weight ratio was reduced by 60% in the mutants of both the young (Figure 2D, 3 week old, p-value = 0.003; 8 controls and 5 mutants were analysed) and mature groups (Figure 2D, p-value = 0.000, 9 controls and 12 mutants were analysed).

The mutant mice had diminished extramedullary haematopoiesis within the red pulp compartment while white pulp remained largely unaffected (Figure 2B, 2C). FACS analysis showed an almost complete absence of erythrocytes (Ter-119 positive) in the mutant spleens (Figure 2E, 0.69±0.17% in the mutant c.f. 55.7±3.9% in the control spleen, p-value = 0.024; five controls and three mutants were analysed) and in Wt1-mutant bone marrow (Figure 2E, 7.3±3.1% in the mutant c.f. 30.3±4.0% in the control bone marrow, p-value = 0.025; five controls and three mutants were analysed).

An intrinsic defect in the mutant haematopoietic system

Maturation of red blood cells requires erythropoietin (EPO) [30], which is synthesised mainly in the kidney. Furthermore, Wt1 has been shown to transcriptionally activate the EPO gene [31]. To determine whether the defect in erythropoiesis is intrinsic to the haematopoietic system, we cultured the mutant bone marrow cells in a methylcellulose-based system where a complete set of factors for supporting haematopoietic differentiation is provided in the medium. After two weeks in culture, despite the presence of all the required growth factors, the Wt1-mutant bone marrow cells failed to differentiate into the erythrocyte lineage, while the control bone marrow cells, as expected, did form red blood cells (Figure 2F, 5.0%±1.87% in the mutant compared with 31.3%±9.6% in the control; five controls and three mutants were analysed, p-value = 0.05).

To address whether this defect in erythropoiesis reflects a cell autonomous role for Wt1 in haematopoiesis, we set out to characterise the 1% of bone marrow cells that express Wt1. Using the Wt1-GFP knockin mouse (Wt1Fgfri/+), we FACS sorted GFP positive cells from the bone marrow of Wt1Fgfri/+ mice and cultured them in a methylcellulose-based system. It has been shown previously that some Wt1-expressing cells in the bone marrow express markers characteristic of short-term haematopoietic stem cells (TERT119CD45Mac1ckitSca1+1) [18] but the differentiation potential of these cells was not investigated. Hence we investigated the potential of these Wt1-GFP cells to differentiate to different haematopoietic lineages in culture. First we stained the GFP positive BM cells with a set of haematopoietic stem cell markers (CD150, CD48, and CD244) [32] and showed that approximately 50% of GFP positive BM cells were in the population of oligodendrocyte-restricted progenitors (CD150 CD48 CD244). Before culturing, no GFP-positive cells were positive for Ter-119 or Cd11b and only a few percent of the cells expressed CD45. After two weeks in culture, the GFP-positive cells were able to form Ter119 (red blood cells), CD45 (white blood cells), and CD11b (granulocytes) positive cells (Figure 2G). From this we can conclude that the Wt1-expressing cells are oligodendrocyte-restricted progenitors.

We then set out to test if the reduction of erythrocytes reflected a decrease in the number of erythrocyte progenitors (Pre CFU-E) using the high resolution myeloerythroid progenitor cell staging method described by Pronk et al [33]. Representative flow cytometric profiles are shown in Figure 3. We saw a significant reduction in the % of Pre CFU-E in the mutant spleen (Figure 3, 0.27±0.06 in the controls and 0.03±0.008 in the mutants, p-value = 0.001; 7 control and 8 mutant mice were analysed). Erythrocyte progenitor cells branch from megakaryocyte-erythrocyte progenitors (PreMegE). Another progenitor that branches from PreMegE is the Megakaryocyte progenitor (MegP) which produces platelets. Both MKP and Pre MegE were reduced significantly in the mutant spleen (Figure 3) However, the number of platelets in the circulation was not affected (control platelet number is 380.5±89.9 K/µL and mutant platelet number is 817.5±164 K/µL). Mutant mice did not show any obvious bleeding tendencies. The half life of platelets is about 35 hours [34]. Platelet deficiency may have developed if the mice had survived longer.
Deletion of *Wt1* leads to rapid bone loss

We observed abnormalities of the growth plate in both the tibias and femurs of *Wt1*-mutant mice. The vascular invasion zones were irregular and anaemic (Figure 4A, indicated by arrow). The proliferative zone chondrocytes of the mutant mice were irregular with less surrounding territorial matrix than control mice (Figure 4A). The inner (marrow) surface of the long bone from the mutant mice was ragged compared with control mice (Figure 4B, arrows), suggesting increased bone resorption. We then analysed the bone architecture of femurs, tibias, and spine 9 days after induction of *Wt1* deletion using μCT (Figure 4C). The 3D movie of the trabecular bone loss is shown in Videos S1 and S2. Trabecular bone volume was reduced by 30% in the mutants (Figure 4D), mostly due to a reduction in trabecular number and a small reduction in trabecular thickness. Furthermore, trabecular connectivity was also reduced. Taken together, these changes in bone architecture would be expected to lead to a substantial reduction in bone strength (Figure 4D). The bone loss observed could be due to either reduced bone growth or increased bone absorption. However, bone formation is a relatively slow process, and in view of the rapidity of the phenotype observed here it seemed that increased bone resorption was the more likely cause. We therefore stained sections of the long bones for the osteoclast marker TRAcP and observed dramatically increased numbers of osteoclasts on the bone surface of the *Wt1*-mutant mice (Figure 4E). To test if these bone phenotypes might reflect an intrinsic role for *Wt1* in the osteoclast and osteoblast lineages, we harvested fresh bone marrow cells from the mutant mice, induced *Wt1* deletion by treating the bone marrow cells with 4-OH tamoxifen, and supplemented with M-CSF and RANKL to induce osteoclast differentiation. Surprisingly and in contrast to the mutant bone marrow cells in which *Wt1* had been deleted by tamoxifen treatment were less capable of forming osteoclasts by differentiation. Immunostaining of a podocyte marker synaptopodin; scale bar, 10 μm. E, EM studies show the presence of foot process (arrows) of the podocytes in control mice (left) while the foot process is completely abolished in the mutants (right) at day 10 postinjection. F, At day 5 post-injection, effacement of foot process starts to show in the mutants c.f. the normal controls; scale bar, 2 μm.

Fat reduction following *Wt1* deletion

The *Wt1*-mutant mice also displayed reduction in the size of fat pads. In addition to the abdominal fat pads which mainly comprise white adipocytes, interscapular brown adipocytes were also atrophic and had fewer lipid cytoplasmic vacuoles than controls (Figure 5A–5J). Although the trend of fat loss was consistent in mutant mice, the reduction of fat pad size seemed to be more variable in the older group of animals (13 weeks, Figure 5K, arrows). In some mutant animals, the reduction in the size of fat pads was observed in both the interscapular and abdominal fat pads, while in other mutants the lipid vacuole size reduction was seen in the abdominal fat pads but not in the interscapular fat pads. The weight of fat pads in the mutant mice did not reflect their actual size because of the oedema (data not shown), and we therefore analysed fat pad volume using whole body μCT scans. Mice were scanned at the start (before tamoxifen injection) and the end of the experiment (9 days after induction). Results from the μCT scan confirmed the substantial fat loss in the mutants (Figure S7, arrows). There was no difference in the number of apoptotic and proliferating cells in the fat pads between mutant and control mice. Histological analysis of the adipose tissues showed that the reduction in the size of fat pads reflected a decrease in the vacuole size of the adipose tissues, as seen in the abdominal fat pads [Figure 5L–5M, *p*<0.05; three controls and three mutant mice were analysed]. Consistent with this loss of fat, there was a significant reduction in the level of AP2 expression in mutant adipose tissue (Figure 5N, *p*<0.05; three controls and three mutants were analysed). *Wt1* expression in fat has not been reported previously. However, here we show that *Wt1* is expressed in the mesentery, inducing osteoblastic differentiation, we observed that *Wt1*-mutant osteoblasts had reduced bone differentiation ability as levels of the osteoblast marker enzyme alkaline phosphatase were reduced (Figure 4G, *p*-value = 0.037; three separate experiments were performed). These results suggest that *Wt1* plays an intrinsic role in both osteoclast and osteoblast differentiation, and that the loss of *Wt1* is likely to disturb bone homeostasis.

**Table 2.** Urine and serum biochemistry analysis of adult mice deleted for *Wt1*.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Urine protein (mg/dl)</th>
<th>Serum urea (mmol/l)</th>
<th>Serum Creatinine (mol/l)</th>
<th>Serum albumin (g/l)</th>
<th>Serum amylase (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 5</td>
<td>Control 4</td>
<td>5.1 ± 1.81</td>
<td>9.63 ± 1.13</td>
<td>25.98 ± 1.78</td>
<td>560.7 ± 64.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutant 3</td>
<td>4.6 ± 0.26</td>
<td>8.1 ± 1.56</td>
<td>22.4 ± 2.55</td>
<td>597.3 ± 41.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.857</td>
<td>0.533</td>
<td>0.267</td>
<td>0.533</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>Control 6</td>
<td>5.62 ± 0.88</td>
<td>11.82 ± 1.08</td>
<td>21.42 ± 2.23</td>
<td>427.3 ± 96.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutant 5</td>
<td>11.08 ± 4.22</td>
<td>15.25 ± 3.51</td>
<td>Not detected</td>
<td>367 ± 34.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.021*</td>
<td>0.127</td>
<td>n/a</td>
<td>0.289</td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>Control 5</td>
<td>65.1 ± 15.2</td>
<td>6.66 ± 0.78</td>
<td>12.08 ± 0.76</td>
<td>25.85 ± 2.27</td>
<td>648 ± 117</td>
</tr>
<tr>
<td></td>
<td>Mutant 3</td>
<td>1503.7 ± 137.3</td>
<td>30.17 ± 8.47</td>
<td>58.6 ± 51.33</td>
<td>15.4 ± 0.57</td>
<td>392 ± 172</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.05*</td>
<td>0.025*</td>
<td>0.064</td>
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</table>

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epididymal, and retroperitoneal fat pads, but not at detectable levels in the abdominal fat pad nor in the interscapular brown adipose tissue (Figure 5O, 5P). Given the fact that adipocytes and osteoblasts have a common origin in the bone marrow, we speculated that Wt1 loss might lead to a disturbance in this population which can be quantified using an antibody to Stro-1. We did in fact find a significant (five fold) increase in this population of cells following Wt1 loss (Figure 4H, p-value = 0.02; four controls and four mutants were analysed).

As adipocytes and osteoblasts arise from the stromal mesenchymal population in the bone marrow, we speculated that Wt1 loss might lead to a disturbance in this population which can be quantified using an antibody to Stro-1. We did in fact find a significant (five fold) increase in this population of cells following Wt1 loss (Figure 4H, p-value = 0.02; four controls and four mutants were analysed).

Deletion of Wt1 leads to atrophy in the exocrine pancreas

Figure 6G–6I (arrows) shows the successful depletion of Wt1 expression in the pancreatic mesothelium. The pancreas from the mutant mice was severely atrophied. H&E staining demonstrated that there was a substantial amount of cell loss in the exocrine tissues while the endocrine pancreas remained largely unaffected (Figure 6A, 6B). Acini in the mutant pancreas were loosely packed and acinar cells appeared atrophied and presented less eosinophilic cytoplasmic staining, suggesting a reduced zymogen content. Residual acinar epithelial cells were rounded and less cohesive with neighbouring cells. Similar aberrant histology started to appear at day 7 afterCre activation (Figure S4B). We saw an increase in the number of apoptotic cells in the mutant pancreas (Figure 6C, 6D) while the number of proliferating cells remained unchanged (Figure 6E, 6F). Although the pathology of our model shares many similarities to pancreatitis mouse models, there was no elevation of serum amylase (Figure 6E, 6F). The phenotypes of adipocytes and in Wt1-mutant mice were observed low-grade inflammation in much of the pancreas and scattered foci of more severe active inflammation. In the Wt1-mutant pancreas, the presence of infiltrating macrophages was confirmed by staining with macrophage marker F4/80 (Figure S6E, S6F); however, staining of CD11b, Gr1, and CD3 were absent (data not shown). Both insulin and amylase expression were present in normal in the mutant pancreas sections (Figure S6A–S6D).

To try to gain more insight into the origin of the pancreatic phenotype, we examined more closely the cell types that express Wt1 in the exocrine pancreas. Pancreatic stellate cells (PSCs) have been implicated in pancreatic inflammation. The expression of Wt1 was examined in adipocytes by staining with macrophage marker F4/80 (control, black; mutant, yellow). FACS analysis of FACS-sorted GFP positive bone marrow cells (from Wt1-GFP knockin mice) before and after grown in a methylcellulose-based medium, where Wt1 is induced in vitro by culturing with 4-OH tamoxifen (1 μM). G, FACS analysis of Ter119, CD45, and CD11b on FACS-sorted GFP positive bone marrow cells (from Wt1-GFP knockin mice) before and after grown in a methylcellulose-based system.

Discussion

The multiple organ disturbance observed in adult mice deleted for Wt1 is striking, and, we believe, unprecedented in terms of severity and rapidity of onset. There is perhaps no need to point out that most of these phenotypes have relevance for diseases common in adults, even though our starting point was a gene more typically associated with embryonic or neonatal development. Our study shows that Wt1 plays a key role in regulating the production or turnover of red blood cells, bone and fat in the adult. Despite intensive analysis of Wt1-null foetuses, including those surviving to 18 days gestation, no developmental defects in these tissues were found previously [4,29]. Thus our study...
contributes to the growing body of evidence that adult tissues may employ different or additional players compared to foetal development. Wt1 is among a list of genes whose methylation increases with age in a genome-wide CpG island methylation profiling study [41]. Therefore Wt1 expression levels may well decrease with age. It will be important to determine whether Wt1 levels in these key cell populations reduce during aging or under different environmental influences. If so, this could contribute to disease-related phenotypes described here.

Although there is much future work needed to elucidate the mechanisms underlying these phenotypes, there are several conclusions we can draw at present. Perhaps, surprisingly, we could detect no significant changes in serum cytokine levels, arguing that the phenotypes we observe are unlikely to be due to a systemic inflammatory response, even though this is often associated with damage to the tissues that are affected in the Wt1 mutant mice. As we argue below, the phenotypes involving the kidney and erythrocytes reflect an intrinsic function of Wt1 in

![Figure 3. High-resolution fractionation of erythroid progenitors in mutant spleen.](image)

Spleen cells were stained with antibodies against Sca-1, c-kit, CD41, CD150, FcgR, CD105, and a cocktail mixture of mature blood cell lineage markers (Lineage). Cells were also stained with 7-AAD and only live cells are displayed. Representative flow cytometric profiles are illustrated. The percentage of MkP, Pro Ery+CFU-E, Pre MegE, and Pre CFU-E in control and mutant spleens is listed.

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Figure 4. Deletion of Wt1 leads to rapid bone loss. A, H&E staining show defects in the Wt1-mutant growth plates (arrows; injected at 3 week old). B, H&E sections of long bone from control and mutant mice (injected at 3 week old). C, uCT images of trabecular bone of femurs from mutant (right) and control mice (left) injected at 10 weeks old. D, Bone histomorphometry analysis on tibia, femur, and spine. Values are expressed as % of change from control mice (8 mutants and 8 control mice were analysed). BV/TV: percentage trabecular bone volume; Tb.Th: Trabecular thickness; Tb.Sp: Trabecular spacing; Tb.N: Trabecular number; Conn.Dn: Connectivity density. *:p<0.05; **:p<0.01; ***:p<0.001. E, TRAcP staining (red) showing osteoclasts in the bone section. F, Analysis of *in vitro* osteoclast formation ability from control and mutant bone marrow cells in the presence of RANKL at various concentrations (10 and 30 ng/ml). G, Analysis of alkaline phosphatase activity in osteoblasts, differentiated from bone marrow cells. H, FACS analysis of % of Stro-1 positive cells in control and mutant bone marrow.

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Figure 5. Fat reduction following Wt1 deletion. Skin pulps from control (A) and mutant (B) mice (injected at 3 week old); scale bar, 1 cm. (C,D) Images of abdominal fat pads; scale bar, 5 mm. H&E staining of the corresponding fat pads is shown in G–J, respectively; scale bar, 25 μm. K, H&E sections of abdominal fat pads from mice injected at 13 week old (arrows indicate lipid vacuoles). Box plot of lipid vacuole size measurement of adipocytes in the abdominal fat pads from the younger group of mice (L) and from the matured group of mice (M). N, Quantitative PCR analysis of AP2 expression in the abdominal fat pads in control and mutant mice. O, Quantitative PCR analysis of Wt1 expression in control and mutant mice.
these tissues or their progenitors. On the other hand, we believe loss of fat and bone is likely to be a combination of systemic and local factors.

The phenotypes involving the haematopoietic system and bone, have their origins wholly or partly within the bone marrow itself. Wt1 is expressed in a restricted haematopoietic progenitor

Figure 6. Deletion of Wt1 leads to atrophy in the exocrine pancreas. (A, B) H&E staining show massive atrophy in the exocrine pancreas. Immunohistochemistry analysis show active caspase 3 (C,D; scale bar, 40 μm); Ki67 (E,F; scale bar, 100 μm) and Wt1-antibody (G,H). Nuclei are stained with DAPI (blue); scale bar, 100 μm. Higher magnification images are shown in I&J scale bar, 20 μm. K, Double immunofluorescence staining of pancreas sections with Wt1-antibody (green)and desmin antibody (red). Nuclei are stained with DAPI (blue); scale bar, 25 μm. Area circled in (K) is shown in a higher magnification in L&M; scale bar,10 μm. N, Double immunofluorescence of Wt1(green) and desmin (red) in cultured PSCs; scale bar 50 μm.

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population and its loss leads to disturbance in red blood cell and osteoclast production. This is consistent with the previous finding that Wt1 expression is upregulated during early myeloid differentiation (particularly in the common myeloid progenitors and megakaryocyte-erythroid progenitors) [18]. In keeping with this, we found the levels of PreMegE, MkP, and Pre CFU-E were significantly decreased in mutant spleen. Given the association of Wt1 with AML, we might have expected an imbalance in the myeloid compartment. Preliminary analysis has not demonstrated a reduction in the absolute number of monocytes and granulocytes in the circulation of Wt1 mutant mice. However, this may have only become evident if the mice had survived longer.

The bone loss in most part is likely to result from the increase in osteoclasts that we observed in the bone marrow. Paradoxically, mutant mice showed a reduction in osteoclast formation ability in vitro. The bone marrow compartment in which we saw an increase in the number of osteoclasts consists of a mixed population of cells. The mesenchymal stromal cells and haematopoietic stem cells are involved in the regulation of osteoclast differentiation, and it is possible that the reduction in osteoclast formation ability is due to a change in the microenvironment.

Figure 7. Cytokine, adipokine, and growth hormone analysis in control and mutant plasma. A. Cytokine profiling in control and mutant plasma. B. Cytokine array performed using plasma from mouse treated with LPS. C. Results from adipokine array showing fold of difference in the level of adipokines between mutant and control mouse plasma. D & E. H&E staining of adrenal glands (control = left, mutant = right; scale bar = 100 μm). F & G. H&E staining of pituitary glands; scale bar = 500 μm. H & I. H&E staining of pituitary anterior lobes; scale bar = 50 μm. J. Measurement of growth hormone (GH) in mouse plasma using ELISA. doi:10.1371/journal.pgen.1002404.g007
in close proximity in the bone marrow and there is known to be
crosstalk between these cell types [42,43]. Our in vitro osteoclast
generation cell culture system started with a restricted population of
cells (bone marrow stromal cells). The in vitro and in vivo
difference could be due to factor(s) that are present in the bone marrow but
absent in the in vitro culturing system.

However, we also found that Wt1 is required for osteoblast
synthesis in bone marrow culture pointing to a role in the
mesenchymal lineage. Consistent with this, our preliminary
experiments have shown that non-haematopoietic Wt1-GFP
positive cells from the bone marrow stroma are able to
differentiate to bone and fat (unpublished observations). Furthermore,
we show here that Wt1 loss also leads to an increase in Srt1
positive stromal mesenchymal stem cells, which may explain partly
the disturbance in adipocyte and osteoblast production in the bone
marrow. Our serum protein analysis showed a dramatic reduction of
IGF-1 levels and this might be expected to contribute to the
bone loss phenotype. Interestingly, deletion of IGF-1 specifically in
the liver, the major source of synthesis, only leads to a 75%
reduction in circulating IGF-1 levels and there is no apparent
phenotype [44]. However, mice that are double homozygous
mutant for IGF-1 and the binding protein acid labile subunit
(ALS) [45] show an 85% reduction in IGF1- levels and a similar
degree of bone thinning to that seen in our Wt1 adult knockout
mice. Hence, it seems reasonable to conclude that the 85%
reduction of IGF-1 levels in our mutant mice is a major factor
behind the bone phenotype. In the Wt1 mutant mice, the IGF-1
levels are much lower than those observed when IGF1 is deleted
specifically in the liver, so either Wt1 is required for IGF-1
expression in non-hepatocytes, or for factors that stabilise IGF-1 in
the serum. Growth hormone, produced by the pituitary gland, is a
major regulator of IGF-1 levels. One possibility was that the
reduction in IGF-1 level was due to defects in the pituitary axis
and downregulation of GH. However, we detected no pathological
abnormalities in the pituitary and adrenal glands, and if anything
GH levels were increased.

Obesity is a major health problem and there is considerable
topical interest in the factors that regulate fat levels. Loss of Wt1
not only leads to reduced adipocyte production in the bone
marrow but also to rapid systemic loss of fat, with dramatically
reduced vacuole size. There are several reasons why we believe
this fat loss is not due to under-nourishment. Fat vacuole reduction
was already apparent 7 days after tamoxifen injection, at which
time the health status of the animals was normal. Nine days post-
injection, the mutant mice still actively sought food and their
stomachs were full on autopsy. Importantly, there was no change
in the levels of circulating leptin, adiponectin, TNF-α, and AgRP/
FIAB, all of which would be expected to change dramatically after
one or two days of calorific restriction. There was a reduction in the
level of lipocalin 2 in mutant serum (Figure 7C). Lipocalin 2 is
abundantly produced from adipocytes [46,47]. The reduction of
lipocalin 2 could be caused by the reduced volume of adipose
tissues in mutant mice. Taken together our findings provide
evidence that Wt1 may influence both the formation and
maintenance of adipocytes. The fat loss is extremely rapid and
given that Wt1 only appears to be expressed in a proportion of fat
pads affected, it seems likely that systemic factors might be
involved. We found that the levels of circulating FGF21 increased
by 3.5 fold in the mutant animals and this would be expected to
induce some fat loss [48].

The RT-PCR result showed that Wt1 expression was detected
in fat pads (Figure 5O). In preliminary experiments to address
whether this reflects expression in mature adipocytes or the
stromal vascular compartments, we digested and fractionated fat
tissues from the Wt1-GFP knockin mice into the floating mature
adipocyte layer and the stromal vascular fraction. The majority of
the GFP signal was seen in the stromal vascular fraction
(unpublished data). This supports the idea that systemic or local
paracrine factors dependent on Wt1 are regulating adipocyte
homeostasis.

The effect of Wt1 loss on bone and fat turnover is interesting in
the context of Wilms' tumours. We and others have shown that the
15–20% subset of Wilms' tumours arising through WT1 loss are
more likely to be stromal (mesenchymal) predominant and often
contain ectopic tissues, including bone, fat, cartilage and muscle
[49,50,51]. Taken together this and our new findings underline the
key role of the mesenchyme and Wt1 in tissue turnover and
maintenance.

With regard to the pancreatic atrophy, this does not appear to be
typical pancreaticitis as there was no increase in serum z-
amylase. However, as discussed above, amylase level may have
increased if the mice had lived longer. Serum cytokine profiles
showed that there was no systemic inflammatory response in the
mutant mice. In line with this there was no observable pathology
in liver, lung and intestine, all tissues susceptible to inflammation.
It remains to be seen whether the severe pancreatic atrophy is due
to loss of Wt1 function within the tissue itself. We can exclude an
effect through loss of Wt1 function in the islet or acinar cells as
deletion of the gene specifically in these cell types using PDX1-Cre
did not lead to overt pathology in the pancreas or elsewhere (P.
Hohenstein, V. Brunton, M. Frame, O. Samson and N. Hastie
unpublished observations). One possibility is that the pancreatic
atrophy arises through activation of the sub-population of stellate
cells that express Wt1 although further study is required to
investigate this hypothesis. Activated stellate cells produce
cytokines [52] and we speculate that these may be responsible
for destroying the acinar cells. Given the published data on foetal
liver [8], the parallel between pancreatic and hepatic stellate cells,
and the role of Wt1 in generating vascular progenitors from the
epidermidium by EMT [5], we hypothesise that a proportion of
pancreatic stellate cells arise from the mesothelium, via an EMT,
once more pointing to the role of this tissue as a source of
mesenchymal progenitor cells.

Despite the accumulating knowledge about the importance of
Wt1 at multiple stages of kidney development, the function of Wt1
in the podocytes of mature glomeruli has remained the subject of
some speculation. Even though children and adult mice with Wt1
mutations characteristic of Denyss-Drash and Frasier syndrome
develop glomerulosclerosis, it was always possible that the damage
had its origin in utero, rather than reflecting a continued function
for Wt1 in the maintenance of the adult kidney. Our results
provide the first evidence that Wt1 is crucial for maintaining the
integrity of mature podocytes. Our model allowed us to test
whether the glomerulosclerosis we observed arises through
abnormalities of cell proliferation or the differentiation state of the mature podocytes. We did not see major changes in proliferation or apoptosis in the mutant glomeruli deleted for Wt1 (using proliferation marker anti-phosph-histone H3 and apoptosis marker active caspase 3, Figure S0E–S0H). However, we showed that loss of Wt1 expression resulted in damage to the foot processes of the podocytes therefore causing a morphological alteration. Nephrin is necessary for the renal filtration barrier and is also a known downstream target of Wt1 during kidney development [33]. Consistent with this we found that nephrin expression levels reduce dramatically after Wt1 deletion, indicating that the transcriptional regulation of nephrin by Wt1 continues into adult life. Here we show that Wt1, known to be a key regulator of nephrogenesis, is also vital for the maintenance of adult glomerular structure and function, something that has been the subject of speculation but not proven until now.

Clearly these findings should be followed up using tissue specific Cre lines. However, at present suitable Cre lines are not available for several of the crucial lineages we wished to investigate, including the mesothelium and mesenchymal stem cells. In the mean time, we have been able to use cultures to show that several of the phenotypes we observed are intrinsic to the bone marrow.

The results presented in this study open new avenues of research into mesenchymal cell function in adult tissues. The cell types that express Wt1 in adult tissues e.g. the hepatic and pancreatic stellate cells and bone marrow progenitors are mesenchymal. The other major cell types expressing Wt1, namely the podocytes and mesothelia are considered epithelial, but are unusual in expressing high levels of mesenchymal markers, such as vimentin. Given our findings, it is interesting to speculate on the possible relationships between the cell types expressing and requiring Wt1 in these different tissues. Different reports have shown that stellate cells may arise from the mesothelium and bone marrow [10,54]. Our studies suggest that Wt1 may have a function in both stellate cells and bone marrow mesenchymal stem cells. Stellate cells, like the epicardioid-derived cells requiring Wt1, synthesise retinoic acid. One of the striking features of stellate cells is the presence of vitamin A (retinoid) droplets and this becomes lost upon stellate cell activation. In the epicardium we have shown that RALDH2 levels and RA are reduced when Wt1 is deleted and that RALDH2 is a direct transcriptional target of Wt1 [9]. We have shown that Wt1 is required for the EMT that generates RA-synthesising coronary vascular progenitors from the epicardium and it is interesting that an EMT is required for activation of stellate cells. It is also notable that stellate cells synthesise high levels of fat and it will be interesting to see if the Wt1 expressing cells in fat have similarities to stellate cells and mesenchymal bone marrow cells.

Finally our findings may also have implications for cancer therapy. There is a growing number of studies developing anti-WT1 immune therapy for common cancers predicated on the belief that Wt1 is expressed at high levels in cancers [20,55], but very low levels in the normal adult. Our findings raise questions about this approach as damage to these normal Wt1-expressing tissues might have adverse effects.

### Materials and Methods

**Generation of Wt1-conditional knockout mice**

Mice were housed and bred in animal facilities at the MRC HGU and the University of Edinburgh. Animals were kept in compliance with Home Office regulations. The Wt1-conditional line was made in our group [5]. To obtain [CAGG-CreER<sup>TM</sup>, Wt1<sup>loxP/loxP</sup>] positive, Wt1<sup>loxP/loxP</sup> null mice we bred [CAGG-<del>CreER</del>TM, Wt1<sup>loxP/loxP</sup>]<sup>−/−</sup> transgenic mice, [CAGG-<del>CreER</del>TM, Wt1<sup>loxP/loxP</sup>] transgenic mice, and the resulting offspring intercrossed. Wt1-GFP knockin mice used in this study were kindly provided by Professor H Sugiyama [10].

**Tamoxifen-induced Wt1 deletion in [CAGG-<del>CreER</del>TM, Wt1<sup>loxP/loxP</sup>] mice**

Cre recombinase was induced by intraperitoneal administration of tamoxifen (4 mg/40 g body weight for 5 days; Sigma). All animal work was carried out under the permission of license. To delete Wt1 in vivo, cells were treated with 4-OH tamoxifen (1 μM, Sigma) for three days.

Full methods are described in Text S1. Antibodies and primers are listed in Tables S1 and S2.

### Supporting Information

**Figure S1** PCR testing for Cre-mediated recombination in inducible Wt1-KO. Top panel: PCR bands show the Cre-mediated recombination in all the tissues tested in the mutant mice (indicated by ‘recombination site’, CreER<sup>TM</sup>−/+; Wt1<sup>loxP/loxP</sup>). The Cre-mediated recombination is not 100% as PCR bands represent no-recombination are still found in the mutant tissues (indicated by ‘lox site’). Lower panel: no recombination was detected in the control mice (tamoxifen injected litter mates, CreER<sup>TM</sup>−/−; Wt1<sup>loxP/loxP</sup>). (PDF)

**Figure S2** Immunohistochemistry analysis of deletion of Wt1 expression in tissues from the mutant mice. Images of sections from mutant mice (CreER<sup>TM</sup>−/−; Wt1<sup>loxP/loxP</sup>) which have also been injected with tamoxifen are at the right column, and images from the control mice (litter mates, CreER<sup>TM</sup>−/−; Wt1<sup>loxP/loxP</sup>) which have also been injected with tamoxifen are at the left column. Using a Wt1-specific antibody, Wt1 expression is detected in the mesothelial lining of organs including pancreas, spleen, lung, and uterus (brown, indicated by arrows). Wt1 expression is not detected in the corresponding tissues from the mutant mice. Scale bars, 20 μm in the heart, pancreas, and spleen. 100 μm in lung, and 40 μm in uterus. (PDF)

**Figure S3** Immunohistochemistry analysis indicate the intactness of the mesothelium in the mutant mice. Images of sections from the mutant mice are shown in the right column and images from the control mice are shown in the left column. Mesothelium lining of organs is detected using a cytokeratin antibody. Scale bars, 20 μm in the heart, pancreas, and spleen. Scale bar, 100 μm in the lung and kidney. (PDF)

**Figure S4** Characterisation of phenotypes in Wt1-KO mice at day 7 post-injection. H&E staining of sections from Wt1-KO mice. A, In the mutant kidney, protein casts are already visible. B, Moderate level of atrophy is seen in the mutant pancreas. C, The reduction in the size of fat vacuoles in the abdominal fat pad is already evident in the mutant mice. D, There is a slight reduction of the size of fat vacuoles in the brown fat pad from mutant mice; scale bars, 50 μm. (PDF)

**Figure S5** Minor gonadal defects in Wt1-KO mice. A, H&E staining show control (left) and mutant (right) testes. B, H&E staining of ovaries from control (left) and mutant mice (right). Follicles and corpora lutea are present in all mice but there was less luteal tissue in the mutant ovaries. In addition, there were fewer large, antral and atretic follicles in the mutant ovaries. Although the size of the gonads appear to be smaller in the
mutants, the difference in the weights (e.g. testes) is not significant. Partial depletion of Wt1 expression in the Sertoli cells in the mutant testes (D) compared with the control (C). Wt1 staining in the granulosa cells in the ovaries (E) and its complete absence in the mutant (F). (G-I), Immunohistochemistry analysis of the expression of Sdmg1 (marker for Sertoli cells), Ptx1 (marker for spermatogonia), and Mvh (marker for spermatogonia, spermatocytes, and round spermatids) between control (left column) and mutant (right column) testes; scale bar, 50 μm. (PDF)

**Figure S6** Immunohistochemistry analysis of markers in the pancreas. Images of sections from the mutant mice are shown in the right column and images from the control mice are shown in the left column. A–D, Immunohistochemistry staining indicate normal insulin and α-amylase expression in the mutant pancreas; scale bar, 50 μm. E, F, Using a pan marker for macrophages (F4/80), infiltrating macrophages are detected in the mutant pancreas; scale bar, 40 μm. (PDF)

**Figure S7** Fat reduction in mutant Wt1-KO using μCT. Representative transverse images taken from the CT scanned mice before and after tamoxifen injection. Control mice (CreERTM/CreERTM, Wt1loxP/loxPloxP), used for fat analysis are the sexed matched littermates of the mutant mice (CreERTM/CreERTM, Wt1loxP/loxPloxPloxP). Light grey shades indicate fat tissues which are present in both control and mutant mice before tamoxifen injection (arrows). Darker shades indicate soft tissues and black shades indicate skeletons. Gaps indicate gastric gas trapped in the intestines of the animal. After 9 days of tamoxifen injection, a reduction in the fat pads is noticed in the mutant mice. (PDF)

**Figure S8** IHC staining of apoptosis and proliferation markers in Wt1-KO mice. A, B, IHC staining of active caspase-3 in control (left) and mutant (right) spleen; scale bar = 50 μm. C, D, IHC staining of phospho-histone H3 in control (left) and mutant spleen (right); scale bar = 100 μm. E, F, IHC staining of active caspase-3 in control (left) and muttanit kidney (right); scale bar = 50 μm. G, H, IHC staining of phospho-histone H3 in control (left) and mutant kidney (right); scale bar = 50 μm. expression in the mutant pancreas; scale bar, 50 μm. (PDF)

### Table S1 Antibodies and dilution factor.

(PDF)

### Table S2 Sequences of primers and Roche Universal Probe Library number used for Q-PCR.

(PDF)

### Text S1 Supporting methods.

(DOC)

### Video S1 Movie shows μCT scanned structures of trabecular bone of femurs from tamoxifen-injected control mouse. (MOV)

### Video S2 Movie shows μCT scanned structures of trabecular bone of femurs from tamoxifen-injected mutant mouse. (MOV)

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## Author Contributions

Conceived and designed the experiments: Y-YC NH KS RvH NB-V. Performed the experiments: Y-YC DB HM EF DS NM AT RB RvH NB-V. Analyzed the data: Y-YC NH DB KS EF W-CL RvF NM DS NB-V. Maintained the mouse samples for EM studies. Performed the experiments: Y-YC DB HM EF DS NM AT RB RvH NB-V. Conceived and designed the experiments: Y-YC NH KS RvH NB-V. Wrote the paper: Y-YC NH RvH. Maintained the animal colony: PH.

## References


