Visceral and subcutaneous fat have different origins and evidence supports a mesothelial source

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Fuelled by the obesity epidemic, there is considerable interest in the developmental origins of white adipose tissue (WAT) and the stem and progenitor cells from which it arises. Whereas increased visceral fat mass is associated with metabolic dysfunction, increased subcutaneous WAT is protective. There are six visceral fat depots: perirenal, gonadal, epiparietal, retroperitoneal, omental and mesenteric, and it is a subject of much debate whether these have a common developmental origin and whether this differs from that for subcutaneous WAT. Here we show that all six visceral WAT depots receive a significant contribution from cells expressing Wt1 late in gestation. Conversely, no subcutaneous WAT or brown adipose tissue arises from Wt1-expressing cells. Postnatally, a subset of visceral WAT continues to arise from Wt1-expressing cells, consistent with the finding that Wt1 marks a proportion of cell populations enriched in WAT progenitors. We show that all visceral fat depots have a mesothelial layer like the visceral organs with which they are associated, and provide several lines of evidence that Wt1-expressing mesothelium can produce adipocytes. These results reveal a major ontogenetic difference between visceral and subcutaneous WAT, and pinpoint the lateral plate mesoderm as a major source of visceral WAT. They also support the notion that visceral WAT progenitors are heterogeneous, and suggest that mesothelium is a source of adipocytes.

Although there has been much progress recently in identifying adult WAT progenitors1–5, the embryological origins of different WAT depots remain obscure. In a recent review this was highlighted as one of the big unanswered questions in the field6. Transcriptomic analysis of pre-adipocytes isolated from the stromal vascular fraction (SVF) of fat has revealed consistent differences in developmental gene expression between visceral depots and between visceral and subcutaneous fat7–9. However, in one study of human depots it was concluded that pre-adipocytes of mesenteric WAT had an expression profile closer to that of subcutaneous than omental pre-adipocytes. Similarly, transcriptome analysis of mouse adipose depots showed that the expression of some developmental genes was high in subcutaneous and perirenal WAT, but low in mesenteric WAT. It has been posited, from these and other similar studies, that each WAT depot could be considered a separate mini-organ. It was not possible to infer either a common or distinct origin for subcutaneous and visceral WAT or the different WAT depots.

The Wilms’ tumour gene, WTI, is a major regulator of mesenchymal progenitors in the developing kidney and heart10,11. During development WTI expression is restricted mainly to the intermediate mesoderm, parts of the lateral plate mesoderm and tissues that derive from these including kidney, gonads, peri-/epicardium, spleen, mesentery, omentum and the mesothelial layer that lines the visceral organs and the body cavity (peritoneum). There is a striking relationship between these Wt1-expressing tissues and the location of the six visceral fat depots. Recently we showed that Wt1 is expressed in several visceral fat depots, but not in subcutaneous WAT or brown adipose tissue12 (BAT). These observations led us to reason that some visceral fat may arise from Wt1-expressing cells during development. To investigate this, we carried out cell lineage analysis.

Before carrying out lineage analysis, it was first necessary to demonstrate that Wt1 is not itself expressed in adipocytes. Adipose tissue is composed of a mixed population of cells, including the mature adipocytes and the SVF. We isolated adipose depots from Wt1-green fluorescent protein (GFP) knock-in mice and separated the tissues

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into mature adipocytes (floating) and the SVF by centrifugation. We showed that the Wt1-positive cells are not present in the floating mature adipocytes (Fig. 1a–d) but they were abundant in the SVF from epididymal, mesenteric, retroperitoneal, omental, epicardial and perirenal WAT (Fig. 1e–l). We stained the floating layer with a lipid dye (LipidTox) and showed that all cells in this layer are LipidTox positive (Supplementary Fig. 1a). Wt1-positive cells were not found in the SVF obtained from subcutaneous WAT or BAT (Fig. 1e–l). We showed previously by quantitative PCR (qPCR) that Wt1 messenger RNA is expressed in all of the visceral fat depots analysed, including epididymal, mesenteric and retroperitoneal WAT. Our fluorescence-activated cell sorting (FACS) analysis reinforces these findings and also revealed Wt1-expressing cells in omental, epicardial and perirenal WAT. We also analysed Wt1 expression in human adipose samples. As for mice, expression was observed in visceral (omentum) WAT but levels were very low or undetectable in whole subcutaneous adipose tissue, and in subclavicular subcutaneous adipose enriched for BAT (confirmed by high levels of UCP-1 expression compared with neighbouring white adipose tissues (an approximately 1,687-fold increase of UCP-1 in the BAT; Supplementary Table 1 and Fig. 1m,n).

For cell lineage analysis, knock-in mice expressing tamoxifen-inducible Cre recombinase at the Wt1 promoter locus as described were crossed with the reporter;mTmG mice. Before Cre-mediated recombination, Tomato is expressed ubiquitously under a pCA promoter. After Cre-mediated loxP recombination that excises Tomato, membranous GFP is expressed. Thus, this system allows genetic marking of CreER-expressing cells at the time of tamoxifen injection (driven by Wt1 promoter activity), and the subsequent tracing of these cells and their progeny. To determine whether adipose tissues may arise from Wt1-expressing cells during development, maternal injection of tamoxifen was performed at embryonic day (E)14.5 (one dose) and the tissues were analysed when the mice were 1.2 years old (n = 4). Approximately 77% of mature adipocytes were Wt1-positive lineage positive in the epididymal fat (Fig. 2a,b). Similarly, large numbers of adipocytes were positive in all visceral fat pads analysed including epicardial (66%), omental (47%), mesenteric (28%), retroperitoneal and perirenal fat depots (Supplementary Fig. 2). However, there were no positive adipocytes derived from Wt1-expressing cells in the subcutaneous WAT and BAT (Fig. 2c,d).

We were interested in determining whether the subcutaneous and visceral fat have different origins and when the separation might take place. During development, Wt1 is first expressed at E8.5 in the intermediate and lateral plate mesoderm in mice. By E14.5, the formation of the body cavity has already taken place. To determine whether cells that are positive for Wt1 expression during E8.5–E14.5 can contribute to the subcutaneous or BAT adipose-lineages, we used a constitutively active Wt1Cre;R26RYFP model. No adipocytes that arise from Wt1-expressing cells were detected in subcutaneous WAT (Fig. 2f), whereas as expected adipocytes in visceral WAT that derived from Wt1-expressing cells were seen (Fig. 2e).

To determine whether any visceral fat may arise from Wt1-expressing cells postnatally during the growth phase, we induced the Cre by oral administration of tamoxifen to three-week-old Wt1CreERT2; mTimG mice (n = 3). GFP expression in adipose tissues was analysed when mice were three months old. Membranous GFP signals were detected (while negative for endogenous Wt1) in epididymal fat pads using an anti-GFP antibody (Fig. 2g, indicated in red). A GFP signal was detected in some cells with mono-ocular lipid vacuoles, characteristic of mature adipocytes. Sections were co-stained with an anti-perilipin antibody (an adipocyte marker, indicated in green) to verify that the GFP-positive cells are adipocytes (Fig. 2h). No GFP signals were detected in the subcutaneous or BAT fat pads (Fig. 2i,j). To show that the absence of GFP signal was not due to the fixing method, we were able to stain the subcutaneous fat deposit with an anti-red fluorescent protein (RFP) antibody (Supplementary Fig. 2f). In addition, we also investigated whether any Wt1-positive cells contributed to adipocytes in bone marrow. Surprisingly, we did not detect any Wt1-positive cells in the bone marrow when mice were induced at embryonic stage (E14.5) or adult (Supplementary Fig. 1b,c).

Given the contribution of juvenile and adult Wt1-expressing cells to mature visceral adipocytes we next examined whether Wt1-positive cells express the cell surface marker sets that characterize progenitors. Recently, two cell populations with the properties of adipocyte progenitors and pre-adipocytes have been isolated by FACS (refs 1,4); these are Lin−CD29+CD34+Sca1−CD24+ and Lin−CD29−CD34+Sca1−CD24− populations, respectively (Fig. 3a). In subcutaneous WAT it was shown that the CD24-negative pre-adipocyte population arises from the CD24-positive progenitor population and there is a shift from the latter to the former after birth4. We show that most of the Lin−Wt1+ cells in the adult adipose depots are in the Lin−CD31−CD29+CD34+ population (Fig. 3b,c, ~90%). In addition, most of the Lin−Wt1+ cells are in the Lin−CD31−CD29+CD34+Sca1+CD24− population (from 60 to 90% depending on fat pad, Fig. 3c); and a smaller percentage of the Lin−CD31−Wt1+ cells is in the Lin−CD31−CD29−CD34+Sca1+CD24+ population, the highest level being in the omentum (Fig. 3c). Figure 3d shows the percentage of cells in each population that are Wt1 positive. Epicardial and omental fat have the highest percentage of cells that are Wt1-positive in both the Lin−CD31−CD29−CD34+Sca1+CD24− (60% and 40% respectively) and the Lin−CD31−CD29−CD34+Sca1+CD24+ (20% and 40% respectively) populations. Both omental and epicardial fat pads are particularly implicated in human disease risk16,17. We demonstrated that the Wt1-positive cells in the SVF are capable of differentiating to adipocytes and muscle in vitro (Supplementary Fig. 3a,b). They have limited ability to form osteoblasts (Supplementary Fig. 3c). Interestingly, we also noticed a difference in the osteoblast-forming ability of SVFs between different fat pads (Supplementary Fig. 3d). SVFs from mesenteric, subcutaneous and epicardial fat pads have a stronger ability to differentiate to osteoblasts compared with SVFs from epididymal and omental fat pads.

To determine whether Wt1 has a role in regulating the behaviour of adipose progenitors, we deleted Wt1 using a ubiquitous tamoxifen-inducible model. In this model, one allele of the Wt1 locus is floxed by loxP sites and the other allele is a GFP knock-in that disrupts Wt1 function (CAGGCreERT2; Wt1loxp/GFP). We performed the deletion in 3-week-old mice and collected the tissues 7 days after the first dose of tamoxifen (n = 4 for each group). We saw a trend of reduction in the percentage of GFP cells in the Lin−CD31−CD29−CD34+Sca1+CD24− population in all fat pads analysed; however, the difference was
Figure 1. Wt1-positive cells reside in the stromal vascular fraction (SVF) in visceral WAT but not subcutaneous WAT or BAT depots. (a–d) Using the Wt1–GFP mouse model, representative FACS plots show that Wt1-positive cells (GFP) are not detected in the mature adipocytes in the epididymal (a), mesenteric (b), subcutaneous (c) or BAT (d) fat pads. (e–l) In the SVF, Wt1-positive cells are found in the epididymal (e), mesenteric (f), retroperitoneal (g), omental (h), perirenal (i) and epicardial (j) depots; however, Wt1-positive cells are not detected in the SVF from BAT (k) or subcutaneous (l) fat pads. GFP signal is indicated on the x axis. Gates are chosen using cells from Wt1–GFP-negative mice (n = 3). (m) The level of WT1 mRNA (normalized with 18S ribosomal RNA) in human visceral and subcutaneous fat is measured by qPCR (y axis is expressed in arbitrary units). V indicates visceral (omental fat in this case) and S indicates subcutaneous fat. (n) The level of WT1 mRNA from human BAT and adjacent WAT is measured by qPCR. Sample 4V is visceral fat, which acts as a positive control (y axis is expressed in arbitrary units).

Statistically significant only in epididymal (P = 0.015, Fig. 3e) and omental fat (P = 0.042, Fig. 3e), but not in the mesenteric and epicardial fat (Fig. 3e). Although there was a reduction in the percentage of Lin<sup>−</sup>CD31<sup>−</sup>CD29<sup>−</sup>CD34<sup>+</sup>Sca1<sup>+</sup>CD24<sup>−</sup> cells that were GFP positive in the epididymal and epicardial fat, this was not statistically significant.
Figure 2 Extensive long-term contribution of the Wt1-positive cells, induced at E14.5 to mature adipocytes in epididymal WAT but not in subcutaneous WAT or BAT. Some Wt1-positive cells in the adult adipose tissues can contribute to mature adipocytes in visceral but not subcutaneous WAT or BAT. One dose of tamoxifen was given to pregnant animals at E14.5 and mice were collected at 1.2-years old (n=3). Sections from various fat pads are stained with GFP antibody (red), Wt1 antibody (green) and DAPI (blue).

(a,b) Epididymal fat pad. 
(c,d) Absence of Wt1-lineaged cells or endogenous Wt1-expressing cells in the subcutaneous (c) and BAT (d) fat pad. 
(e,f) Immunostaining of sections of fat dissected from the constitutively active Wt1\textsuperscript{Cre}; R26RYFP, indicating the presence of the GFP-positive adipocytes (indicated in green) in the visceral fat (e) and absence of GFP-positive cells in the subcutaneous WAT (f). 
(g) Wt1-positive cells in the epididymal fat pad from the Wt1\textsuperscript{CreERT2};mTmG model induced at 3 weeks old gave rise to mature adipocytes (GFP, red; endogenous Wt1, green; cell nuclei, DAPI, blue) when mice were collected at 3 months old. 
(h) Mature adipocytes are indicated by perilipin antibody staining (GFP, red; perilipin, green; cell nuclei, blue). 
(i,j) Absence of GFP-positive cells in the subcutaneous (i) or BAT (j) fat pad. 
(k) The mesothelium structure in adipose tissue arises from Wt1-positive cells (red). 
(l) Mesothelium in adipose tissues (epididymal) express endogenous Wt1 (indicated in green) and is Wt1-lineage positive (indicated in red).

As for other visceral organs that are covered by a layer of Wt1-expressing mesothelium, the visceral adipose depots were also found to be lined by a GFP-positive mesothelium. Figure 2k shows epididymal fat at 6.5 months following tamoxifen induction at 4 months where GFP is indicated in red and the adipocyte marker perilipin is marked in green. This mesothelial layer still expressed endogenous Wt1 and GFP 1.2 years after being induced at E14.5 (Fig. 2). We also observed a Wt1-positive and cytokeratin-positive mesothelial layer in the other visceral fat depots (Supplementary Fig. 4a). The importance of the mesothelium as a source of mesenchymal progenitors for interstitial cells/structures during development has been demonstrated in the heart, lung, gut and liver\textsuperscript{10,15,18-23}. Furthermore, the peritoneum and its extensions, the mesentery and omentum, are essentially mesothelial structures that develop fat depots. Therefore, we reasoned that the mesothelium might be able to contribute to the adipocytes that often physically
Figure 3 Wt1-positive cells in adult adipose tissues express adipose progenitor surface markers. (a) A schematic representation of the FACS strategy taken from ref. 1. (b) Dot plots showing representative FACS staining profiles and gating of adipose SVF from adult Wt1–GFP mice. Lin-negative and CD31-negative populations (P8) from live singlets are selected. The Lin–CD31– population is further separated on the basis of expression of CD34 and CD29. The CD34 and CD29 double-positive cells are further analysed on the basis of the expression of Sca1 and CD24. Most of the Wt1–GFP-positive cells (indicated in green) are in the Lin–CD31–CD34+CDCD29C population. (c) The percentage of each population in Lin–GFP+ from different fat pads is shown n=4; data represent the mean ± s.e.m. (d) The percentage of the cells in each population that are Wt1–GFP positive. Epi, epididymal; Mes, mesenteric; RP, retroperitoneal; OM, omentum. n=4; data represent the mean ± s.e.m. (e) Effect of deleting Wt1 on the percentage of different populations of adipose progenitors. FACS analysis of percentage of adipose progenitors in fat pad from 3-week-old female WGER mice (CAGGCreERT2.Wt1loxP=GFPlittermates that were injected with tamoxifen and collected at day 7 post-injection (n=4 for each group; data represent the mean ± s.e.m and one-tailed Student’s t-tests were used to assess statistical significance. *P<0.05). Cre-negative (CAGGCreERT2.Wt1loxP=GFplittermates injected with tamoxifen are included as the control.

attach to the visceral organs. Consistent with this idea, in the lineage tracing model, a short pulse of tamoxifen (induced at E14.5 and analysed at E16.5) labelled only mesothelial cells at the site where future fat pads will be formed (Supplementary Fig. 4b). Before postnatal day 4, adipocytes are not yet formed in the thin, translucent membrane-like structure adjacent to the testes that later becomes...
the epididymal fat pad\textsuperscript{24}. The authors of ref. 24 developed an \textit{ex vivo} culture system for this epididymal appendage and showed that it can produce adipocytes, following the normal time course of fat production \textit{in vivo}. Furthermore, they showed that if this structure was removed \textit{in vivo}, epididymal fat was no longer produced. We reasoned that this structure looks very similar to a mesothelium and hence it would express Wt1. Therefore, we cultured this layer to see whether it expresses Wt1, a major marker for the mesothelium, and to determine whether Wt1-expressing cells contribute to mature adipocytes. Using pups from the Wt1\textsuperscript{CreERT2, mTmG} line, this layer of mesothelium was dissected from postnatal day 3 pups and cultured \textit{ex vivo} in medium containing 4-OH tamoxifen and adipogenic medium. Time-lapse video analysis showed that all cells in the mesothelium expressed GFP at the beginning and no GFP signals were detected. After 5.5–9.5 h, many cells expressed GFP and some of these started to migrate out from the mesothelium (Supplementary Video 1). No GFP cells were detected in the Cre-negative mesothelium from the Wt1\textsuperscript{CreERT2, mTmG} pups (Supplementary Video 2). After 110 h, some cells that migrated out started to produce lipid droplets. The mesothelial nature of the epididymal appendage was confirmed by immunohistochemistry using mesothelin antibody (Supplementary Fig. 5a). Wt1–GFP embryo stained with the mesothelin antibody was used as a positive control (Supplementary Fig. 5b).

We then used multiphoton microscopy to further characterize the lipid-droplet-containing cells in the centre of the explant culture. Lipid droplets generate strong coherent anti-Stokes Raman scattering (CARS) signals (indicated in red). The explant was imaged on day 1, day 7 and day 14 after culturing with adipogenesis medium. It is clearly seen that there are Wt1-derived cells from the epididymal appendage (that is, cells with membranous GFP signal) that are capable of generating lipid containing adipocytes (Fig. 4a). We confirmed that the lipid-droplet-filled cells were adipocytes by staining with FABP4 and perilipin antibodies (Supplementary Fig. 5c,d). The results obtained from both the explant culture system and \textit{in vivo} lineage tracing in adult mice show there are Wt1-derived and non-Wt1-derived adipocytes. We were interested in this heterogeneity and in determining whether the Wt1-derived adipocytes (that is GFP positive) differ from the other adipocytes (RFP positive). In the \textit{ex vivo} system (Fig. 4b), preliminary results suggested that Wt1-lineage adipocytes had fewer but larger lipid droplets per cell compared with RFP-positive adipocytes. Similarly, in the \textit{in vivo} epididymal fat there were fewer droplets in the GFP-positive cells than the RFP-positive cells but there was no significant difference in droplet size. The cell size and percentage of lipid content did not differ between GFP-positive or RFP-positive adipocytes either in the \textit{ex vivo} or \textit{in vivo} systems (Fig. 4d).

We reasoned that mesothelium might be a source of visceral adipocytes and hence we investigated whether mesothelium during development shares the cell surface marker expression pattern of adipose progenitors and pre-adipocytes seen in the adult fat pads. FACS analysis was carried out on GFP-positive mesothelial cells removed by gentle collagenase treatment (Fig. 5b). We chose E14.5 as a starting point as this is the stage at which embryos were induced in the previous lineage tracing analysis (Fig. 2). At E14.5, the mesothelium from Wt1–GFP embryos is Lin\textsuperscript{−}CD31\textsuperscript{−}CD34\textsuperscript{+}CD29\textsuperscript{+}CD24\textsuperscript{+}Sca1\textsuperscript{+}. The expression pattern remains unchanged at E15.5 and E16.5. There is an emergence of Lin\textsuperscript{−}CD31\textsuperscript{−}CD34\textsuperscript{+}CD29\textsuperscript{+}CD24\textsuperscript{+}Sca1\textsuperscript{+} population of cells at E17.5. At postnatal day (P)1 and P7, there was a reduction in cells with CD24-positive expression and the population of Sca1-positive cells increased. In adult, most cells are Lin\textsuperscript{−}CD31\textsuperscript{−}CD34\textsuperscript{+}CD29\textsuperscript{+}Sca1\textsuperscript{+}CD24\textsuperscript{+} (Fig. 5a). Hence, the mesothelium expresses cell surface markers characteristic of WAT progenitors and pre-adipocytes and there is a gradual shift in the pattern that broadly resembles that described for subcutaneous WAT (ref. 4).

To confirm the mesothelial nature of the cells used for the FACS analysis, we used qPCR to measure the mRNA levels of two validated mesothelial markers, Msln and Upk3b (ref. 25), in E14.5 and adult preparations. The levels of Msln and Upk3b mRNA were approximately two orders of magnitude higher in the GFP-positive cells than in the GFP-negative cells from the collagenase-treated layer at both E14.5 and adult stages (Supplementary Table 2a). Importantly, the GFP-positive cells after a brief pulse of tamoxifen in the lineage tracing system, and GFP-positive cells isolated from the epididymal appendage used for explant showed similar absolute levels of mesothelial marker mRNA expression to the validated mesothelia taken at E14.5 and adult (Supplementary Table 2b,c).

One major conclusion from this study is that visceral and subcutaneous WAT, the so-called bad and good fat, have different origins during development. At least 30–80% (depending on the depot) of visceral adipocytes in 14-month-old mice have arisen from cells that express Wt1 between E14.5–16.5 (the likely duration of action of tamoxifen). Given that only a single dose of tamoxifen has been administered, Cre-mediated recombination is unlikely to be complete and some fat progenitors will arise outside this time frame, these percentages are likely to be underestimates. Taken together, these data suggest that Wt1 marks true long-term adipocyte progenitors late in gestation. We find no contribution of Wt1-expressing cells to subcutaneous WAT or BAT during embryogenesis or in postnatal life in the fat pads analysed. This conclusion is also supported by experiments using a constitutive Wt1\textsuperscript{Cre}\textsuperscript{lox} for the lineage analysis. The question arises as to the exact location and nature of the Wt1-expressing adipose progenitors/pre-adipocytes. Over the past few years, it has become evident that the mesothelium is an abundant source of mesenchymal cells that contribute to a variety of tissues and cell types\textsuperscript{10,15,18–21}. Here we provide evidence that the mesothelium is a source for at least some visceral WAT progenitors/pre-adipocytes. First, we show that visceral WAT has a Wt1-expressing mesothelial layer. Second, in the region where visceral fat will form a short pulse of tamoxifen at E14.5 in the lineage tracing model predominantly labels mesothelial cells as revealed by immunohistochemistry and qPCR on sorted cells. Third, using an \textit{ex vivo} culture system, we show that an epididymal mesothelial appendage can produce adipocytes from Wt1-expressing cells. Finally, Wt1-positive mesothelial cells express cell surface markers characteristic of WAT progenitors late in gestation and this shifts postnatally to a cell surface marker pattern characteristic of pre-adipocytes, thus approximating a scenario reported for subcutaneous WAT (ref. 4). The mesothelium has its origins in the lateral plate mesoderm and we propose that this is the source of some adipose progenitors in all visceral WAT depots. Some visceral WAT may also arise from Wt1-expressing cells derived
Figure 4 Characterizing an ex vivo model of mesothelium and epididymal appendage differentiation into adipocytes using multiphoton microscopy. (a) Multiphoton microscope images showing epididymal appendage explants (WT1<sup>CremERT2</sup>; mTmG/+) cultured in adipo genesis differentiation medium at day 1, day 7 and day 14. Membranous GFP signal is indicated in green. CARS is used to detect lipid (indicated in red). An enlarged image for day 14 is also included. (b,c) Lipid droplet analysis of the WT1-derived adipocytes (green) and the neighbouring adipocytes (red) from the explants culture system (b) or from epididymal fat pad induced by tamoxifen in vivo (c). Green, GFP; red, tdTomato; cyan, CARS. (d) Analysis includes quantification of number of lipid droplets per cell, quantification of lipid droplet diameter, quantification of cell size, and quantification of the percentage of lipid content per cell. Horizontal lines represent mean with standard deviation. Statistics source data for d can be found in Supplementary Table 5.

from the intermediate mesoderm. BAT has been shown to arise from paraxial mesoderm and, consistent with these different mesodermal origins, we find no contribution of WT1-expressing cells to the BAT lineage. This leaves open the question of the developmental origins of subcutaneous fat. It seems reasonable to propose that the source will be outside the peritoneum. The diagram in Fig. 5c explains these conclusions and speculations.

A second conclusion from the lineage tracing is that WT1 contributes to a subset of adipocytes and that the proportion varies between visceral depots. Support for this idea of progenitor
heterogeneity comes from a previous study\textsuperscript{26} that showed that 50% of retroperitoneal fat arises from myf5-expressing cells but at most only a few per cent of epididymal adipocytes arise from this source. This is in agreement with our demonstration that most epididymal fat arises from Wt1-positive progenitors, whereas fewer than 50% of retroperitoneal adipocytes derive from Wt1-positive progenitors. In the adult, most Wt1-positive cells in the SVF express markers characteristic of pre-adipocytes. The proportion of pre-adipocytes expressing Wt1 varies from 10 to 60% between depots, again supporting the idea of heterogeneity. Similarly, depending on the depot, Wt1-positive cells comprise 4–40% of the adult progenitor population, being most abundant in omental and epicardial fat. All of this raises the interesting question of whether the adipocytes arising from Wt1-positive progenitors/pre-adipocytes have different properties from the adipocytes arising from the Wt1-progenitors/pre-adipocytes. Our first experiments to address this issue using multiphoton microscopy on \textit{in vivo} and \textit{ex vivo} epididymal fat suggest that the adipocytes arising from Wt1 progenitors/pre-adipocytes have fewer droplets. More work will be required to determine whether the Wt1-derived adipocytes have different metabolic properties from the non-Wt1-derived adipocytes. Our preliminary data suggest that Wt1 is not only a marker for visceral fat progenitors but may also function in their regulation. More work will be needed in mice and in culture to investigate this and the mechanisms involved.
METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Y-Y.C., R. Bandiera, A. Serrels, O.M.M.E., R.M.C. and N.H. Performed the experiments: Y-Y.C., R. Bandiera, A. Serrels, M.L., R.H.S., B.R.W. and R.M.C. Wrote the paper: Y-Y.C. and N.H.

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METHODS

Mice and tamoxifen-induced lineage tracing. Mice were housed and bred in animal facilities at the MRC HGU, University of Edinburgh, and the University of Nice. Animals were kept in compliance with Home Office regulations. The tamoxifen-induced lineage tracing model was made by crossing Wt1CreERT2 with the mTmG reporter line. The tamoxifen-induced Wt1 deletion model, where one copy of the Wt1 locus is a GFP knock-in and the other allele (exon 1) is flanked by loxp sites, was made in our group ([CAGG-CreERT2-positive, Wt1lox/lox]). Wt1-GFP knock-in mice used in this study were provided by H. Sugiyama (Osaka University School of Medicine, Japan). The constitutively active Wt1-CAGG line used in this study was described previously. The crosses of the Wt1CreERT2;R26TdyFPFP were made in the University of Malaga.

To induce lineage tracing in adult mice, tamoxifen was gavaged to animals at 8 mg per 40 g body weight twice per week for two consecutive weeks. Tamoxifen dosing at E14.5 was performed by maternal gavaging at 8 mg per 40 g body weight for one dose. Fostering of pups from maternally tamoxifen-dosed females is required.

Mice used in this study were adult (aged between 2 and 10 months if not specified). Male mice were used in the experiments unless otherwise indicated.

Human subjects. Human subcutaneous (abdominal) and visceral (omentum) adipose biopsies were obtained from patients undergoing abdominal surgery. Paired brown and white adipose tissue samples were obtained from the superficial (SAT) and deeper (BAT) supraclavicular region from euthyroid patients undergoing elective thyroid or parathyroid surgery. Ethical committee approval and written informed consent were obtained.

RNA extraction. RNA from adipose tissue was extracted using TriZol following the manufacturer’s protocol.

Quantitative PCR. The same protocol is described in ref. 12. The primer sequence and corresponding Roche Universal Probe are listed in Supplementary Table 3.

Immunohistochemistry. The same protocol for immunostaining is described in ref. 12. All primary antibodies were applied overnight at 4 °C. Secondary antibodies are purchased from Molecular Probes. The antibodies used are listed in Supplementary Table 4.

FACS analysis. Adipose tissue was made into a single-cell suspension by dissecting into small pieces (~1–2 mm²) before being digested using collagenase (2 mg ml⁻¹, Invitrogen) in HBSS (containing 4 mg ml⁻¹ BSA) for one hour at 37 °C. The cell suspension was allowed to stand for 5 min before centrifuging at 1,200 g before being stained for surface antibodies. Antibodies are from eBioscience unless otherwise stated. The list of antibodies is included in the Supplementary Material. For mulicolour analysis and sorting, colour compensation samples were produced by singly staining SVF cells with one antibody of each in the combined fluorochromes as well as by Fluorochrome Minus One controls. FACS analysis and sorting were performed using a FACS flow cytometer and data were analysed using FlowJo and FACSDiva (BD) software.

FACS analysis on floating mature adipocytes. Mature adipocytes from fat pads of male adult mice were separated from the SVFs by collagenase digestion followed by passing through 70–100 μm filters. After centrifugation, mature adipocytes floated on the top layer were collected. Adipocytes were fixed with 0.01% PFA for 10 min, and deeper (BAT) supraclavicular region from euthyroid patients undergoing elective thyroid or parathyroid surgery. Ethical committee approval and written informed consent were obtained.

In vitro myogenic, osteogenic and adipogenic differentiation. SVF cells from fat pads of Wt1CreERT2;R26TdyFPFP mice were cultured in DMEM (10% FCS) before plating out for differentiation. For myogenic differentiation, SVF cells were expanded in DMEM (10% FCS and 1 mM 4-OH tamoxifen). Later, C2C12 myoblasts were co-cultured with SVF cells to induce differentiation (DMEM containing 3% FCS and 1 mM 4-OH tamoxifen) for two weeks. Wt1-lineaged myotubes were visualized by immunostaining of GFP and desmin antibodies. For osteogenic differentiation, SVF cells from fat pads of Wt1CreERT2;R26TdyFPFP mice were expanded in DMEM (10% FCS and 1 mM 4-OH tamoxifen) before sorting using a panel of adipose progenitor surface markers. Sorted cells were cultured in osteoblast differentiation medium for three days before staining for alkaline phosphatase. For adipogenesis, SVF cells were expanded in DMEM (10% FCS, penicillin/streptomycin and 1 mM 4-OH tamoxifen) before changing to
adipogenesis differentiation medium. Fat pads from both male and female adult mice were used.

Statistical analysis. All results are expressed as mean ± s.e.m. Student’s t-test was used to determine $P$ values. Statistical significance was defined as $P < 0.05$. No statistical method was used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment. Representative images showing reproducible results were used based on experiments performed using more than three animals in each repeated experiment, unless otherwise stated.
Supplementary Figure 1. Representative FACS analysis of LipidTox staining from the floating layer from adipose tissue digestion. (a) Single cells (P1) from the floating layers were stained with LipidTox (Deep Red) or without and analysed by FACS (n=3). (b) Immunofluorescence of bone marrow from Wt1-Cre<sup>ERT2</sup> mTmG mice (induced at E14.5 and harvested at one-two month old). Adipocytes in the bone marrow were stained with perilipin antibody (indicated in red; cell nuclei were stained with DAPI in blue). No GFP+ cells were detected. (c) FACS analysis of bone marrow cells. No GFP+ cells were detected. Bone marrow cells from Cre-negative mice were used for the GFP gating (n=3).
**Supplementary Figure 2** Immunofluorescence images of adipose tissues from lineage tracing study. One dose of tamoxifen was given to pregnant animals at E14.5 and mice were analysed at 1.2 years-old (n=4). Sections from various fat pads were stained with GFP antibody (indicated in red), Wt1-antibody (indicate in green), and cell nuclei were stained with DAPI (blue). a, epicadial; b, omental; c, perirenal; d, retroperitoneal; e, mesenteric. (f) Subcutaneous section stained with anti-RFP antibody (indicated in green) and GFP (indicated in red).
Supplementary Figure 3 Differentiation of SVFs into myotubes, osteoblasts, and adipocytes. (a) Myotube differentiation assay. SVFs from Wt1CreERT2, mTmG mice were co-cultured with C2C12 myoblast and maintained in myotube differentiation medium for two weeks. Cells were stained with GFP (green) and desmin (red) or RFP (red) and desmin (green). Multinucleated myotubes expressing both GFP and desmin or RFP and desmin were observed (yellow). (b) adipocyte differentiation assay. SVFs were cultured in adipocyte differentiation medium for 10 days. GFP signal is indicated in green and RFP signal is indicated in red. Fluorescence images were merged with bright field image. Lipid droplets were seen in adipocytes. (c) Osteoblast differentiation assay. Sorted cells were cultured in osteoblast differentiation medium (DMEM, 10% FCS, P/S, ascorbate-2-phosphate, β-glycerophosphate and dexamethasone for 3 weeks. Alkaline phosphatase assay (blue) was used to detect osteoblasts. Osteoblast differentiation assay which was performed in SVFs sorted from different fat pads was shown in (d).
Supplementary Figure 4  Visceral fat pads are covered by mesothelia and short tamoxifen-pulsing labeled mesothelia. (a) Visceral fat pads in adult mice are covered by mesothelia. Immunofluorescence staining of sections from epididymal, epicardial, mesenteric, and omental fat pads (from Wt1-GFP mice) with GFP (green) and cytokeratin (red, a marker for mesothelia) antibodies. DAPI is indicated in blue. (b) Short tamoxifen-pulsing labeled mesothelia in lineage tracing experiment. Immunohistochemistry using GFP (green) and cytokeratin (red) antibodies on samples from the region of GI tract in Wt1-CreERT2;mTmG embryos induced with tamoxifen at E14.5 and analysed at E16.5. Cell nuclei is stained with DAPI (blue).
Supplementary Figure 5 The epididymal appendage is covered by mesothelia and lipid-filled cells in the explant culture are adipocytes. (a) Whole mount immunostaining of epididymal appendage from postnatal day 2 CD1 pups with a mesothelin (red) antibody. Immunofluorescence image of mesothelin is overlapped with the brightfield image. (b) Immunofluorescence staining of sections from E18.5 Wt1-GFP embryo showing the GFP+ layer (green) covering the surface of GI tract is mesothelin (red) positive. Cell nuclei are stained with DAPI (blue). (c) Lipid droplet-filled GFP+ cells (green) in the epididymal appendage explant culture (Wt1-CreERT2;mTmG) express adipocyte marker (FABP4, red) and (d) perilipin (green). GFP in (d) is indicated in red.
Supplementary Video Legends

**Supplementary Video 1** Time-lapse video of adipocytes formation in a Cre-positive epididymal appendage *ex vivo* culture. Time-lapse of the epididymal appendage in a Cre-positive Wt1-lineage tracing model (Wt1-Cre<sup>ERT2</sup>;mTmG) cultured in matrigel and adipocyte differentiating medium (including 1 µM 4-OH tamoxifen). Cells in the mesothelium expressed RFP at the beginning and then started expressing GFP. Adipocytes became visible in cells with lipid droplets.

**Supplementary Video 2** Time-lapse video of adipocytes formation in a Cre-negative epididymal appendage *ex vivo* culture. Time-lapse of the epididymal appendage in a Cre-negative Wt1-lineage tracing model (Wt1-Cre<sup>ERT2</sup>;mTmG) cultured in matrigel and adipocyte differentiating medium (including 1 µM 4-OH tamoxifen). No GFP cells were detected.