Adipocyte Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based Hematopoietic and Bone Regeneration

Graphical Abstract

Highlights

- A stem cell-like population produces adipogenic and osteogenic lineages in bone

- Aging and high-fat diet specifically promote expansion of the adipogenic lineage

- Multipotent cells promote and adipogenic cells reduce hematopoietic reconstitution

- Adipogenic cells inhibit fracture repair, which is restored by DPP4 inactivation

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In Brief

Ambrosi, Schulz, and colleagues define a stem cell-like population that gives rise to osteogenic progeny and promotes hematopoietic reconstitution. Aging and high-fat diet reprogram the mesenchymal lineage to preferentially give rise to adipogenic cells that impair reconstitution and bone fracture healing. Bone tissue repair is fully restored by DPP4 inhibition.
Adipocyte Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based Hematopoietic and Bone Regeneration

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SUMMARY

Aging and obesity induce ectopic adipocyte accumulation in bone marrow cavities. This process is thought to impair osteogenic and hematopoietic regeneration. Here we specify the cellular identities of the adipogenic and osteogenic lineages of the bone. While aging impairs the osteogenic lineage, high-fat diet feeding activates expansion of the adipogenic lineage, an effect that is significantly enhanced in aged animals. We further describe a mesenchymal sub-population with stem cell-like characteristics that gives rise to both lineages and, at the same time, acts as a principal component of the hematopoietic niche by promoting competitive repopulation following lethal irradiation. Conversely, bone-resident cells committed to the adipocytic lineage inhibit hematopoiesis and bone healing, potentially by producing excessive amounts of Dipeptidyl peptidase-4, a protease that is a target of diabetes therapies. These studies delineate the molecular identity of the bone-resident adipocytic lineage, and they establish its involvement in age-dependent dysfunction of bone and hematopoietic regeneration.

INTRODUCTION

The adipocyte-enriched yellow bone marrow develops during aging and obesity, and it may contribute to a dysfunction of the osteogenic and hematopoietic niches of long bones. According to recent data, increased marrow adipose tissue (MAT) in obese individuals is correlated with osteoporosis and increased fracture risk (Fazeli et al., 2013; Schwartz, 2015). Similarly, type II diabetes has been linked to reduced bone quality (Carnevale et al., 2014). However, a causal relationship has not been fully established, as mice fed a high-fat diet also showed a rapid increase in MAT while bone parameters remained unaffected (Doucette et al., 2015). Nevertheless, marrow adipogenesis is associated with impaired hematopoiesis (Naveiras et al., 2009). Several seemingly opposing influences, such as aging and obesity but also caloric restriction and anorexia, irradiation therapy, thiazolidinediones, and glucocorticoids, promote the accumulation of MAT (Devlin and Rosen, 2015). Two types of MAT have been described: the constitutive MAT (cMAT), localized around the growth plates, and regulated MAT (rMAT), which accumulates later in life and in response to high-fat feeding (Scheller et al., 2015).

It has been proposed that MAT progenitors are radio-resistant, non-hematopoietic cells with a mesenchymal origin (Berry et al., 2015). Mesenchymal stromal cells (MSCs) are capable of giving rise to osteoblasts, chondroblasts, and adipocytes (Fridenshtein et al., 1968). Lineage tracing has linked the developmental origin of MSCs to Osterix1 (Osx1)-expressing cells in neonatal bone marrow (Mizoguchi et al., 2014) and to cells expressing Leptin receptor (LepR) in adult bone marrow (Zhou et al., 2014). Concomitantly, Leptin signaling regulates MAT formation and osteogenesis in adult mice (Yue et al., 2016). Subsets of bone marrow MSCs, such as C-X-C motif chemokine (CXCL)12-abundant reticular (CAR) cells, provide essential maintenance signals for the hematopoietic stem cell niche (Mendelson and Frenette, 2014; Morrison and Scadden, 2014).

The involvement of MAT in local osteogenic and immunomodulatory processes as well as systemic metabolism emphasizes the necessity to further investigate the adipogenic potential of bone marrow mesenchymal cells. Using a combination of flow cytometry and genetic lineage tracing, we delineate considerable cellular heterogeneities, and we identify distinct subsets of mesenchymal cells within the bone marrow cavity of the long bones in male mice. Specifically, we describe a multipotent population with stem cell-like properties residing within the perivascular niche that gives rise to unilaterally committed...
sub-populations of osteochondrogenic and adipogenic lineages under in vitro and in vivo conditions. Adipocytic cells significantly impair hematopoietic repopulation and bone fracture healing. This latter effect is mediated by secreted Dipeptidyl peptidase-4 (DPP4), an important target of anti-diabetes treatments (Lamers et al., 2011; Marguet et al., 2000). These findings altogether support the hypothesis of a deteriorative role of MAT in bone health and hematopoiesis.

Consistent with white adipose tissue (Berry and Rodeheffer, 2013; Rodeheffer et al., 2008), we isolated a population of unilaterally committed adipogenic progenitors that was CD45⁻CD31⁻ Sca1⁺CD24⁻ and a CD45⁺CD31⁻ Sca1⁺CD24⁺ population that displayed tri-lineage differentiation potentials (Figures 1C and 1D). Colony-forming unit (CFU-F) potentials and in vitro recovery rates were highest in fibroblastic cells expressing Sca1 and/or Pa and were highest in the CD45⁺CD31⁻ Sca1⁺Pa⁺CD24⁺.
subset (Figures S1D and S1E). In culture, CD45
 subset of adipogenic cells was likely not initially detected due
 of the tri-potent CD45
 CD45
 CD45
 subset (Figures S1D and S1H). Importantly, two separate clonal analyses of the tri-potent CD45
 CD31
 Sca1
 CD45
 /C0
 within the CD45
 a
 distributed. Further analyses of P
 to the diaphysis, whereas adipogenic progenitors were evenly
 expression (Figures S1K–S1N).

 To test in vivo differentiation potentials, we generated a triple-
 transgenic mouse strain carrying alleles of the Zfp423-EGFP
 reporter, Adiponectin (Adipoq)-Cre, and a luciferase reporter
 within the Rosa26 locus that is only expressed after Cre medi-
 ated recombination, e.g., in mature adipocytes (repAdLuc, Fig-
 ure S2J), and we generated a second strain with constitutive
 red fluorescence (mTmG reporter allele) crossed to the
 Zfp423-EGFP reporter (repAdTom, Figure S2K). Fresh cells of all
 four populations isolated from repAdLuc or repAdTom by fluo-
 rescence-activated cell sorting (FACS) (Figure S2L) were trans-
 planted into the sternal region of B6/Albino mice. After 8 weeks,
 in vivo imaging of luciferase and Perilipin immunofluorescence
 (IF) showed that transplants of the CD45
 CD31
 Sca1
 CD24
 and
 CD45
 CD31
 Sca1
 Zfp423
 adipogenic populations consistently gave rise to bona fide mature adipocytes, while in vitro osteogenic
 CD45
 CD31
 Sca1
 P2
 cells did not (Figures 1F, 1G, S3A, and S3B). Interestingly, CD45
 CD31
 Sca1
 CD24
 cells gave rise to luciferase-positive and -negative transplants
 (Figure 1F). Movat-Pentachrome staining of luciferase-negative
 tissues of CD45
 CD31
 Sca1
 CD24
 and CD45
 CD31
 Sca1
 P2
 cells revealed bone-like osteochondrogenic/mineralized
 structures but never adipocytes (Figures 1G and S3C). Consis-
 tent with this observation, intratibial injections of the four cell
 populations showed that only CD45
 CD31
 Sca1
 CD24
 ,
 CD45
 CD31
 Sca1
 CD24
 , and CD45
 CD31
 Sca1
 Zfp423
 adipogenic cells, but never CD45
 CD31
 Sca1
 P2
 , gave rise to Zfp423
 adipogenic cells in their endogenous microenvironment (Fig-
 ure S3D). Moreover, only CD45
 CD31
 Sca1
 CD24
 transplants
 were able to give rise to adipogenic CD45
 CD31
 Sca1
 CD24
 cells
 (Figure S3E). In summary, these findings reveal a multi-line-
 age potential of the CD45
 CD31
 Sca1
 CD24
 cell population
 that can give rise to a lineage of fate-committed adipogenic pro-
 genitor cells (APCs: CD45
 CD31
 Sca1
 CD24
 ) that, in turn,
 give rise to a more mature pre-adipocyte (preAd: CD45
 CD31
 Sca1
 Zfp423
 ) and that, in parallel, could yield a population of
 unilaterally committed osteochondrogenic progenitor cells
 (OPCs: CD45
 CD31
 Sca1
 P2
 ), under in vitro and vivo condi-
 tions (Tables S3 and S4).

 **Osteo-Adipogenic Cell Populations Derive from a Mesenchymal, Non-endothelial, Non-hematopoietic Lineage**

 To determine the developmental lineages of these four cell
 populations, lineage tracing was performed using mouse strains ex-
 pressing Cre-recombinase under the control of promoters to
 mark hematopoietic (Vav1), endothelial (Cdh5 and Tek/Tie2), or
 mesenchymal (Prx1 and P0) cells, or mature adipocytes (Adipoq)
that were crossed to the mTmG-reporter mouse strain (Berry and Rodeheffer, 2013; Krueger et al., 2014). IF and flow cytometric analysis revealed a non-hematopoietic, non-endothelial, but mesenchymal lineage for the bone marrow–resident cells with purely adipogenic (APCs, adipocytes) or osteogenic (bone lining, osteocytes) potentials, which was also true for the CD45−CD31−Sca1−CD24+ stem cell-like population with tri-lineage potential (Figures 2A–2C).

As shown in previous reports on adipose tissues, CD45+ hematopoietic cells were exclusively traced by Vav1-Cre, whereas CD31+ cells did not trace to the expected Cdh5-Cre endothelial origin but rather to the Vav1-Cre hematopoietic driver (Figure 2D) (Berry and Rodeheffer, 2013). Unexpectedly, only ~50% of marrow adipocytes and CD45−CD31−Sca1−CD24+ APCs were marked by the Pα-Cre driver (Figure 2), contrasting our data from the Pα-EGFP reporter and also from the FACS analyses (Figure S3F), but consistent with previous reports of incomplete recombination by this Cre strain (Krueger et al., 2014; Zhou et al., 2014). Lastly, comparing the developmental lineages of adipogenic progenitors in Prx1-Cre;mTmG mice revealed labeling of inguinal WAT (iWAT) and skeletal muscle–resident CD45−CD31−Sca1− cells that were comparable to bone, while brown adipose tissue (BAT) and epididymal WAT (eWAT) progenitors displayed almost no labeling (Figure S3G). Moreover, gene expression patterns of in-vitro-differentiated progenitors from bone resembled most closely those derived from iWAT, with similar adipogenic differentiation capacity and expression of general adipogenic genes Peroxisome proliferator-activated receptor-γ (Pparg) and CCAAT/enhancer-binding protein-α (Cebpa) and absent or low expression of BAT markers Uncoupling protein-1 (Ucp1) and Cell Death-Inducing DFFA-Like Effector A (Cidea) (Figures S3H–S3M), altogether indicating that the marrow adipocytic lineage is more closely related to white rather than brown adipocytes.

The Adipocytic Lineage Responds to Diet and Aging

We next examined gene expression in femora and tibiae from young (2 months) and old (25 months) mice. Consistent with previous reports (Devlin and Rosen, 2015), expression of adipogenic marker Pparg was increased in old bones. However, adipogenic potential of CD45−CD31−Sca1− progenitors isolated from old bones was unchanged. Conversely, osteogenic marker Osterix (Osx/Sp7) expression was significantly reduced, as was osteogenic capacity of CD45−CD31−Sca1− progenitors isolated from young bones (Figure 3A). Next, mice of both ages were fed a high-fat diet for either 24 hr (1dHFD) or 10 days (10dHFD). Accumulation of MAT was more pronounced in old animals after 10dHFD, while brown adipose tissue (BAT) and epididymal WAT (eWAT) progenitors displayed almost no labeling (Figure S4A). FACS analysis of bone marrows showed that donor-derived CD45−CD31−Sca1−CD24+ APCs were only partially GFP+, indicating that these cells maintained their original identity but also gave rise to maturing Zfp423+ preAds (Figure 4B). In full support of the sternal transplantation data, donor-derived (tdTomato+) adipocytes were only observed when multipotent CD45−CD31−Sca1−CD24+ cells, APCs, or preAds, but not OPCs, were transplanted (Figure 4C). FACS analysis of bone marrows showed that donor-derived progenitors were retained 5 weeks after transplantation, indicating long-term survival (Figure 4D, upper panels). Expression of Zfp423-driven EGFP was readily detectable in all marrows except after OPC transplants. All cells from the Zfp423+ preAd transplants maintained GFP expression, indicating no reversion ability toward GFP− stages, whereas transplants of CD45−CD31−Sca1−CD24+ APCs and Zfp423+ preAds were only partially GFP+, indicating that these cells maintained their original identity but also gave rise to maturing Zfp423+ preAds (Figure 4D, middle panels). Consistent with our initial characterization, Zfp423+ cells no longer expressed Sca1 or CD24 (Figure 4D, lower panels).

Tibiae that had received adipogenic transplants (APCs or preAds) 5 weeks prior to analysis showed significant reductions in cellularity but no change in overall bone marrow chimerism (Figures 4E and 4F). No differences in any of these parameters were observed in contralateral tibiae and regarding donor-derived myeloid and lymphoid cells, blood chimerism, or splenic hematopoietic progenitor cells, as these latter cells may also originate from other, non-injected bone sites (Figures 4E, 4F, and S5A–S5D). Importantly, frequencies of hematopoietic lineage (Lin−) Sca1+c-Kit+ hematopoietic progenitor cells (LSK cells) and repopulation with donor-derived (CD45.1+) CD34− long-term (LT)−LSK and CD34− short-term (ST)−LSK cells were significantly reduced after adipogenic transplants (Figures 4G–4I). A similar trend of impaired hematopoietic reconstitution was observed after transplantation of APCs in a separate long-term reconstitution experiment 16 weeks after irradiation, but it did not reach statistical significance (Figures S5E–S5G). Over the course of long-term reconstitution, no differences in blood cellularity were observed between young and aged mice (Figures 4J and S5H–S5J).
chimerism or blood lymphoid or blood myeloid cells were observed in animals transplanted with APCs (Figures S5H–S5J). FACS analysis revealed that transplanted cells were retained, and it confirmed that only CD45^−CD31^−Sca1^+CD24^+ and APCs gave rise to Zfp423^+ preAds, albeit at markedly lower frequencies (Figure S5K).

Figure 2. Lineage Tracing of MAT Reveals a Mesenchymal, Non-endothelial, Non-hematopoietic Lineage
(A) Representative merged IF images of bone-resident adipocytes from the indicated Cre-mTmG reporter mouse strains (green, EGFP; red, Perilipin; blue, DAPI).
(B) Reporter analysis of bone lining cells and cortical bone-resident osteocytes (blue arrows; green, GFP; red, tdTomato; blue, DAPI).
(C) FACS analysis of tdTomato^+ and GFP^+ cells in the CD45^−CD31^−Sca1^+ population (percentages represent average values).
(D) Quantification of GFP^+ bone-resident adipocytes, bone lining cells, and osteocytes by image quantification and flow cytometric analysis of bone stroma cells populations (n = 3–4 mice/genotype). Mean ± SEM. Scale bars, 10 μm.
See also Figure S3.
**The Adipocytic Lineage Inhibits Bone Regeneration**

To determine the pathophysiological role of the adipocytic lineage during bone healing, all four populations isolated from recipient mice were transplanted into the vicinity of a stabilized tibia fracture and analyzed after 14 days (Figures 5A, upper panels, S6A, and S6B). The μCT quantification showed a significant decrease of total bone mineral density (BMD) at the fracture site following transplantation of adipogenic populations when compared to the no-cell control group (Figures 5A, middle panels, and 5B). Histomorphometric analysis of fracture/callus sites indicated reduced areas of mineralized tissue and increased amounts of cartilaginous tissues following transplantation of the adipogenic populations, e.g., APCs and preAds, compared to all other groups (Figures 5A, lower panels, and S5C–S5E). Due to the lineage restrictions shown in the cell culture and sternal transplants, these observations likely indicate delayed healing and, thus, that the cartilaginous structures are entirely derived from the host in the adipogenic transplant groups. Aside from adopting an adipogenic fate after intratibial injection (Figure S3D), multipotent CD45-CD31-Sca1-CD24+ and the two adipogenic populations produced some fibrous tissue, whereas only multipotent CD45-CD31-Sca1-CD24+ and OPCs contributed to chondrogenic and osteogenic structures (Figures S6C–S6G). These observations indicate a negative regulatory role of adipocytic cells during fracture healing, further establishing the detrimental role of MAT in aged bone homeostasis.

**DPP4 Released from MAT Inhibits Bone Healing**

To identify potential negative regulators of regeneration processes, RNA sequencing (RNA-seq) was used to further characterize the molecular identity of all four populations (Figures S7A and S7B). Principal-component and hierarchical clustering analyses clearly supported the distinct nature of each population, providing a second line of evidence for the lineage restriction of adipogenic commitment of the closely related APC and preAd populations (Figures 6A–6C). Differential expression (DE) analyses produced several sets of known and potential new candidate genes to define each population (Figures 6D–6G; Table S5). For instance, canonical stem cell markers (e.g., *Nog, Il11m*, and *Myc*) were enriched in the CD45-CD31-Sca1-CD24+ multipotent stem cell population (Figure 6D). Moreover, signals known to regulate hematopoietic stem cell (HSC) quiescence and maintenance (e.g., *Cxc112, KitisCf*, and *Vcam-1*), showed the highest expression in this population, along with the highest, but not exclusive, expression level of LepR that was also expressed in the other cells types. The OPC population expressed the classical osteogenic (e.g., *AlpI, Dmp1*, and *Col1a1/2*) and chondrogenic markers (e.g., *Acan, Col2a1*, and *Sox9*), as well as previously described skeletal stem cell markers (*Itgs5* and *Cd200*), at elevated levels (Figure 6E; Table S9) (Chan et al., 2015). The adipogenic populations expressed high levels of markers that have been linked to the adipocytic lineage (i.e., *Cd34, Ebf2*, and *Dpp4*) (Avogaro et al., 2014; Rodeheffer et al., 2008; Wang et al., 2014) or adipocyte differentiation (i.e., *Vim, Ppara*; Figures 6F and 6G) (Franke et al., 1987; Goto et al.,...
Figure 4. Distinct Roles of Progenitor Populations during Hematopoietic Recovery
(A) Schematic depiction of competitive hematopoietic repopulation assay.
(B and C) H&E stains of injected tibiae (B), and tdTomato (red) and Perilipin (green) (C) IF co-localization on adipocytes in injected tibiae (green arrow, host derived; orange arrow, donor derived). Scale bars, 50 μm.
2011). As expected, expression of Zfp423 was highest in Zfp423+ preAds (Figure 6F). Thus, our RNA-seq analysis confirmed the cellular characteristics of the four populations, and it establishes the CD45−/CD31−/Sca1+CD24+ multipotent stem cell population as a population expressing elevated levels of Cxcl12 and Lepr that are important regulators of HSCs and osteogenesis (Greenbaum et al., 2013; Yue et al., 2016).

To identify signals that could mediate the negative effects of adipogenic cells on bone healing, we screened the dataset for secreted factors that were significantly enriched in the adipogenic populations. Among the most significantly regulated secreted factors was the gene encoding for Dipeptidyl peptidase-4 (Dpp4), a protease shed from the plasma membrane that is an important target of clinical diabetes treatments (Figure 7A) (Avogaro et al., 2014; Marguet et al., 2000). Consistent with the RNA-seq data, CD26 (the membrane-bound form of DPP4) was enriched on the surface of adipogenic cell populations, and only CD45−/CD31−/Sca1+CD24+ and APCs, but not OPCs, released DPP4 into the medium after adipogenic differentiation (Figures S7C and S7D).

Expression of Dpp4 was increased in distal tibiae of old mice that contain most ectopic adipocytes, and explant cultures of old tibiae released greater amounts of DPP4 (Figures 7B and 7C). While treatment of CD45−/CD31−/Sca1+CD24+ and APCs with the DPP4 inhibitor sitagliptin had no effect on adipogenesis, it significantly enhanced osteogenic gene expression and mineralization of multipotent CD45−CD31−/Sca1−CD24+ and OPCs during osteogenic differentiation (Figures 7D, 7E, S7E, and S7F). While no positive effect was found in untreated OPC transplants (Figure 5), the improved OPC function following sitagliptin may serve to promote bone healing. Exposure to recombinant DPP4 slightly impaired osteogenic, but did not alter adipogenic differentiation (Figures S7G–S7J). Treatment of mice with two DPP4 inhibitors, Diprotin A and sitagliptin, significantly accelerated tibia fracture healing.
Figure 6. RNA-Seq Defines the Cellular Identities of Bone-Resident Sub-populations

(A–C) The principal-component analysis (PCA; A), correlations scores (B) of the top ten genes driving PC1 and PC2 in (A), and hierarchical clustering analyses (C) of RNA-seq from all four cell populations.

(D–G) Heatmaps of selected differentially expressed (DE) genes, divided by candidates reported in the literature (known, asterisks indicate no significant DE between individual groups) and novel markers, enriched in CD31+CD24* (D), OPC (E), APCs and preAd in (F), and APCs or preAd (G) cell populations. See also Figure S7 and Table S5.

(Figures S7K and S7L), and intraperitoneal (i.p.) injections of sitagliptin for 9 days significantly increased the frequency of osteogenic progenitors while decreasing the frequency of APCs in non-fractured tibiae (Figure 7F). Administration of sitagliptin was sufficient to abolish the negative effects of transplanted adipogenic cells on bone healing while surprisingly promoting bone healing after OPC transplants (Figures 7G–7I). Lastly, transplantation of Dpp4-deficient APCs similarly prevented the inhibitory effects of APCs on fracture healing (Figures 7J–7L).

DISCUSSION

Marrow adipogenesis is a highly regulated process that responds to a variety of endocrine signals, dietary cues, and...
Figure 7. DPP4 Inhibition Reverses the Negative Effects of Adipogenic Cells on Bone Regeneration

(A) Gene expression intensities of Dpp4 from RNA-seq analysis.

(B) Dpp4-mRNA levels in whole proximal and distal tibiae of young (2 months) and old (15 months) male mice (n = 5).

(C) DPP4 secretion by whole tibia explants from young and old mice (n = 4).

(D and E) The mRNA levels (D) of Runx2 and Osterix (Osx/Sp7) and Alizarin Red S staining and quantification (E) of CD45-CD31-Sca1+CD24+ and OPCs either treated with PBS (control, white bars) or sitagliptin (100 mM, black bars) during osteogenic differentiation (n = 3). Scale bars, 30 μm.

(F) FACS analysis of OPC, CD45-CD31-Sca1+CD24+, and APC cell frequencies in tibiae from male mice either i.p. injected with PBS (white bars) or sitagliptin for 9 days (black bars; n = 9–10). Mean ± SEM; *p < 0.05, **p < 0.01, and ****p < 0.0001.

(G–I) Representative Movat-Pentachrome stains (G) of fracture calluses from control PBS-treated mice that received osteogenic (PBS/OPC) or adipogenic (PBS/APC) intratibial transplants and animals treated with sitagliptin for 1 week after fracture and receiving the same transplants of osteogenic (Sita/OPC)
pathologies (Scheller and Rosen, 2014), reflecting the necessity of a highly adaptive reservoir of stem/progenitor cells. Our study establishes a unidirectional developmental hierarchy of the bone marrow adipocytic lineage from a multipotent CD45^-CD31^-Sca1^-CD24^ stem cell-like population toward distinct progenitor cell populations with unilaterally committed osteochondrogenic or adipogenic fates. Our results further suggest that adipogenic progenitors irreversibly mature toward a preAd stage: while APCs can maintain their identity they also give rise to Zfp423^ preAds that cannot revert to Sca1^-Zfp423^ progenitors. This unidirectional process enables the definition of two distinct maturation stages that subsequently differentiate into mature Zfp423^ marrow adipocytes. These findings are consistent with recent studies that have defined the heterogeneity of mesenchymal cell populations in WAT and BAT in some detail (Berry and Rodeheffer, 2013; Gupta et al., 2012; Rodeheffer et al., 2008; Schulz et al., 2011). The adipocytic as well as osteochondrogenic populations of the bone derive from a mesenchymal and non-hematopoietic, non-endothelial lineage. Previous work has also shown that neural crest-derived, Nestin^ cells are the likely developmental origin of the adult mesenchymal cells described here (Isen et al., 2014; Morikawa et al., 2009b; Nagoshi et al., 2008; Takashima et al., 2007).

Our data on developmental lineages, differentiation capacity, and adipocyte phenotype suggest that marrow-resident adipocyte progenitors more closely resemble white, rather than brown, adipogenic cells. This is consistent with previous reports, but it does not rule out specific differences in endocrine function between WAT and BAT (Scheller and Rosen, 2014). However, our RNA-seq analysis shows that several genes enriched in bone-resident preAds are also expressed in committed brown pre-adipocytes (e.g., Ebf2, Entpd2, Fam129a, and Acy3) (Wang et al., 2014), which would support at least some potential similarities to the BAT lineage (Kings et al., 2012).

A high-fat diet rapidly increases expansion of the adipogenic, but not the osteochondrogenic lineage. This induction is more pronounced in aged bone marrow. Whether aging also affects expansive capacities of subsets of adipose tissue-resident progenitors remains to be determined, but our data clearly suggest that this process may be involved in the pathogenic processes related to MAT accumulation. This observation is supported by a report on leptin-mediated regulation of diet-induced adipogenesis in the bone marrow (Yue et al., 2016). Of note, decreased numbers of OPCs after high-fat diet feeding were only observed in young animals, which could be due to leptin resistance as observed in aged animals (Gabriely et al., 2002) but which would also suggest that expansion of adipogenic cells may not be exclusively leptin dependent.

Sca1^ cells, e.g., the multipotent CD45^-CD31^-Sca1^-CD24^ population, as well as APCs, associate to L type vessels that are known to host HSCs (Sivaraj and Adams, 2016) and occur in the vicinity of the endosteum and could, thereby, readily affect the hematopoietic niches or contribute to osteogenic processes. Interestingly, a multipotent differentiation potential of at least partially overlapping populations of bone-resident populations, such as PzS (Sca1^-Pz^) cells, cells expressing LepR, or Nestin-expressing stromal cells, has been described before (Mendez-Ferrer et al., 2010; Morikawa et al., 2009a; Yue et al., 2016; Zhou et al., 2014), but these populations overlap at least partially with several of the populations described here. For instance, almost all LepR^ cells are Pz^ and contain all CD45^-CD31^-Sca1^ cells (Zhou et al., 2014). Our clonal analysis of CD45^-CD31^-Sca1^-CD24^ cells identified this population as a highly homogeneous pool of multipotent cells, indicating additional enrichment in this stem cell-like sub-population. In support of this observation, clonal analysis of PzS or LepR^ cells showed multipotency only in a smaller subset of clones that may have derived from sub-populations expressing CD24 (Morikawa et al., 2009a; Zhou et al., 2014). Importantly, the multipotent stem cell-like population also promotes hematopoietic repopulation and is enriched for cells expressing the hematopoiesis maintenance cytokine CXCL12 (Greenbaum et al., 2013; Zhou et al., 2014). In comparison to the other three cell types defined in our study, we find that CD45^-CD31^-Sca1^-CD24^ multipotent cells also express the highest levels of Lepr and other pro-hematopoietic signals, such as KIt/Scf and Vcam1, and, thus, could represent a further purification step of a mesenchymal stem cell that supports hematopoiesis (Ding et al., 2012; Lewandowski et al., 2010). In summary, while we cannot exclude that several independent adipogenic and osteogenic lineages exist in bone, our study strongly suggests that the CD45^-CD31^-Sca1^-CD24^ cell type, the skeletal equivalent of previously described adipocyte stem cells in WAT (Rodeheffer et al., 2008), can give rise to populations unilaterally committed to either lineage and provides maintenance signals essential for the hematopoietic niche.

Consistent with previous reports (Chan et al., 2015; Steenhuis et al., 2008), we describe a committed OPC population that may at least partially arise from the CD45^-CD31^-Sca1^-CD24^ multipotent stem cells. Comparison of expression signatures suggests these correspond to previously described skeletal stem cells (Chan et al., 2015), which have been proposed to be one of two distinct skeletogenic stem cell populations contributing to post-natal development of bone (Worthley et al., 2015). In our study, Grem1 mRNA was detected in all populations but was highest in the multipotent cells. While Worthley et al. (2015) clearly showed that Grem1^ cells are mostly CD45^-CD31^-Sca1^- skeletal stem cells, a small subset of Grem1^ cells was also Sca1^ and could thus also mark the multipotent stem cell-like population we describe here. Further work is required to determine the extent to which CD45^-CD31^-Sca1^-CD24^ cells contribute to the osteogenic lineages in embryonic and adult stages.

Ectopic adipocyte accumulation in the bone marrow cavity is believed to contribute to age-related impairment of bone or adipogenic (Sita/APC) cells. Fracture callus total volumes (TVs) were analyzed for mineralized callus volume (H, BV/TV) and fibrous tissue volumes (I, FV/TV) (n = 5–7).

(J–L) Representative Movat-Pentachrome stains and histomorphometric analyses (as in G–I) of fracture calluses 14 days after injury from mice that received osteogenic (OPC) or adipogenic (APC) intratibial transplants from either DPP4-KO or wild-type (WT) animals (n = 9). Mean ± SEM; p < 0.05: a, versus PBS or WT/OPC; b, versus PBS or WT/APC; c, versus sitagliptin or DPP4-KO/OPC; and d, versus sitagliptin or DPP4-KO/APC. Scale bars, 100 μm. See also Figure S7 and Table S6.
regeneration and hematopoiesis (Carnevale et al., 2014; Fazeli et al., 2013; Le et al., 2016; Naveiras et al., 2009; Schwartz, 2015). An increased risk for fractures and complications, such as non-unions, is associated with aging- and obesity-induced MAT accumulation (Nuttall and Gimble, 2004). Bone healing is tightly regulated with an initial inflammatory phase, followed by cartilaginous callus formation, the deposition of a fibrous matrix, and subsequent mineralization through osteogenic cells (Einhorn and Gerstenfeld, 2015). We here identify the cellular basis for the pro-adipogenic shift observed during high-fat diet feeding and aging. Cells committed to the adipogenic lineage not only inhibited bone healing but also acute hematopoietic reconstitution. The limited negative effect of the adipocytic lineage during long-term hematopoietic recovery further suggests that the lack of a pro-adipogenic stimulus is beneficial to bone homeostasis. Follow-up studies will have to determine whether these effects are also true for distinct processes, such as bone remodeling as opposed to bone healing, and subtypes of healthy and pathological MAT (Cawthorn et al., 2014; Scheller et al., 2015) and whether sex-specific differences occur as only male mice were analyzed in this study. A potential mediator of such detrimental effects on bone homeostasis is DPP4, as it was recently shown to impair hematopoietic recovery and bone health (Broxmeyer et al., 2012; Kim and Cho, 2016; Monami et al., 2011). Our results provide mechanistic insights into the beneficial effects of DPP4 inhibitors during bone regeneration by targeting the adipocytic lineage, and they potentially link MAT as a source of DPP4 to the pathophysiology of systemic insulin resistance. In summary, we here delineate the ontogeny of MAT and the adipocytic lineage, which exerts negative effects on bone healing and hematopoiesis. These findings suggest that MAT accumulation causes the age-related dysfunction of the bone marrow niches and can be implicated in multiple pathological processes that interfere with appropriate maintenance of bone tissue repair and the hematopoietic system.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2017.02.009.

**AUTHOR CONTRIBUTIONS**

T.H.A. and T.J.S. conceived the study and wrote the manuscript. T.H.A. conducted the majority of the experiments. A. Scialdone, A.G., A.-M.J., L.W., C.B., and S.G. contributed research to this study. A. Schurmann, H.-F., and D.W.L. contributed valuable materials and expertise to the article. L.R.S. conducted experiments and helped write the manuscript.

**ACKNOWLEDGMENTS**

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Mouse Ly-6A/E (Sca-1) APC (Clone: D7) | eBioscience | Cat#: 17-5981 |
| Anti-Mouse Ly-6A/E (Sca-1) Alexa Fluor 700 (Clone: D7) | eBioscience | Cat#: 56-5981 |
| Anti-Mouse Ly-6A/E (Sca-1) APC/Cy7 (Clone: D7) | Biolegend | Cat#: 108125 |
| Anti-Mouse CD45 FITC (Clone: 30-F11) | eBioscience | Cat#: 11-0451 |
| Anti-Mouse CD45 APC (Clone: 30-F11) | Biolegend | Cat#: 103111 |
| Anti-Mouse CD45 PE (Clone: 30-F11) | Bioscience | Cat#: 12-0451 |
| Anti-Mouse CD31 (PECAM-1) FITC (Clone: 390) | eBioscience | Cat#: 17-0311 |
| Anti-Mouse CD31 (PECAM-1) APC (Clone: 390) | eBioscience | Cat#: 17-0451 |
| Anti-Mouse CD140a (PDGF Receptor a) PE-Cyanine7 (Clone: APAS) | eBioscience | Cat#: 12-1401 |
| Anti-Mouse CD24 APC-eFluor 780 (Clone: M1/69) | eBioscience | Cat#: 47-0242 |
| Anti-Mouse CD24 PE (Clone: 30-F1) | eBioscience | Cat#: 12-0241 |
| Anti-Mouse CD26 PerCP-Cyanine5.5 (Clone: H194-112) | eBioscience | Cat#: 45-0261 |
| Anti-Mouse CD45.1 PE (Clone: A20) | eBioscience | Cat#: 12-0453 |
| Anti-Mouse CD45.1 APC (Clone: A20) | eBioscience | Cat#: 17-0453 |
| Anti-Mouse CD45.2 Alexa Fluor 700 (Clone: 104) | eBioscience | Cat#: 56-0454 |
| Anti-Mouse CD11b PE (Clone: M1/70) | eBioscience | Cat#: 12-0112 |
| Anti-Mouse Ly-6G (Gr-1) PE (Clone: RB6-8C5) | eBioscience | Cat#: 12-5931 |
| Anti-Mouse CD19 APC-eFluor 780 (Clone: eBio1D3) | eBioscience | Cat#: 47-0193 |
| Anti-Mouse CD33e APC-eFluor 780 (Clone: 145-2C11) | eBioscience | Cat#: 47-0031 |
| Mouse Hematopoietic Lineage FITC Cocktail (Clone: 17A2, RA3-882, M1/70, TER-119, RB6-8C5) | eBioscience | Cat#: 22-7770 |
| Anti-Mouse CD117 (c-Kit) APC (Clone: 2B8) | eBioscience | Cat#: 17-1171 |
| Anti-Mouse CD34 Alexa Fluor 700 (Clone: RAM34) | eBioscience | Cat#: 56-0341 |
| Anti-Mouse CD16/CD32 PE-Cyanine7 (Clone: 93) | eBioscience | Cat#: 25-0161 |
| Rabbit anti-RFP (tdTomato) | Abcam | Cat#: ab62341 |
| Goat anti-GFP | NovusBiologicals | Cat#: NB100-1770 |
| Rat anti-CD45 | NovusBiologicals | Cat#: NB100-77417 |
| Goat anti-Perilipin | Abcam | Cat#: ab61682 |
| Rabbit anti-Perilipin | Sigma | Cat#: P1873 |
| Rat anti-CD31 | Dianova | Cat#: DIA-310 |
| Rat anti-Sca1 | Abcam | Cat#: ab25195 |
| Rat anti-BrdU | Cedarlane | Cat#: CL2700AP |
| Mouse anti-Aggrecan | Merck Millipore | Cat#: MABT84 |
| Goat anti-Osteocalcin | BioRad | Cat#: 7060-1815 |
| Goat anti-CD24 | Abcam | Cat#: ab202963 |
| Alexa Fluor 488 goat anti-rabbit | Abcam | Cat#: ab150077 |
| Alexa Fluor 488 chicken anti-goat | Life Technologies | Cat#: A-21467 |
| Alexa Fluor 488 donkey anti-rat | Life Technologies | Cat#: A-21208 |
| Alexa Fluor 488 goat anti-mouse | Abcam | Cat#: ab150113 |
| Alexa Fluor 568 goat anti-rabbit | Abcam | Cat#: ab175471 |
| Alexa Fluor 594 donkey anti-rabbit | Life Technologies | Cat#: A-21207 |
| Alexa Fluor 594 donkey anti-goat | Abcam | Cat#: ab150132 |
| Alexa Fluor 680 donkey anti-rabbit | ThermoFisher | Cat#: A10043 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Anti-β-Actin Peroxidase-conjugated | Sigma | Cat#: A3854 |
| Anti-human/mouse UCP1 | R&D Systems | Cat#: MAB6158 |
| Peroxidase goat anti-mouse | Abcam | Cat#: ab97023 |

**Chemicals, Peptides, and Recombinant Proteins**

| Name | Source | Identifier |
|------|--------|------------|
| Calcein | eBioscience | Cat#: 65-0855-39 |
| Propidium Iodide (PI) | Sigma | Cat#: P4170 |
| Recombinant Mouse DPPIV/CD26 Protein | R&D Systems | Cat#: 954-SE |
| Diprotin A (Ile-Pro-Ile) | Sigma | Cat#: I9759 |
| Sitagliptin | biomol | Cat#: Cay-13252-250 |
| Cal-Rite Fixative | ThermoFisher | Cat#: 10599428 |
| Roti-Histofix 4% | Carl Roth | Cat#: P087.3 |
| Sudan Black B | Sigma | Cat#: 199664 |
| Fluormount-G | eBioscience | Cat#: 00-4958-02 |
| Oil Red O | Sigma | Cat#: O0625 |
| Alizarin Red S | Carl Roth | Cat#: A5533-25G |
| Alcian Blue 8GX | Sigma | Cat#: A3157 |
| Crystal Violet | Sigma | Cat#: C0775 |
| BrdU | Sigma | Cat#: B5002 |
| D-Luciferin - K+ Salt Bioluminescent Substrate | Perkin Elmer | Cat#: 122796 |
| MCDB201 Media | Sigma | Cat#: M6770 |
| Dexamethasone | Sigma | Cat#: D-4902 |
| L-Ascorbic acid 2-phosphate | Sigma | Cat#: A8960 |
| Insulin-transferrin-selenium (ITS) mix | Sigma | Cat#: I3146 |
| Linoleic acid-Albumin | Sigma | Cat#: L9530 |
| Epidermal growth factor | PeproTech | Cat#: 315-09 |
| Leukemia inhibitory factor | MerckMillipore | Cat#: ESG1107 |
| Platelet-derived growth factor BB | PeproTech | Cat#: 315-18 |
| Basic fibroblast growth factor | Sigma | Cat#: F0291 |
| Indomethacin | Sigma | Cat#: I7378 |
| Recombinant Human Insulin | Roche | Cat#: 11376497001 |
| Isobutylmethylxanthine | Sigma | Cat#: I5879 |
| 3,3',5-triiodo-L-thyronine (T3) | Sigma | Cat#: T6397 |
| β-glycerophosphate | Sigma | Cat#: G9891 |
| L-thyroxine | Sigma | Cat#: T0397 |
| Transforming growth factor β1 | PeproTech | Cat#: 100-21 |

**Critical Commercial Assays**

| Name | Source | Identifier |
|------|--------|------------|
| DPP4 ELISA | ThermoFisher | Cat#: EMDP4 |
| RNeasy Plus Micro Kit | QIAGEN | Cat#: 74034 |
| SMARTer PCR cDNA Synthesis kit | Clontech | Cat#: 634925 |
| Advantage 2 PCR kit | Clontech | Cat#: 639207 |
| Bioanalyzer DNA High-Sensitivity kit | Agilent Technologies | Cat#: 5067 |
| Nextera XT DNA Sample Preparation Kit | Illumina | Cat#: FC-131 |
| Nextera Index Kit | Illumina | Cat#: FC-131 |

**Deposited Data**

| Name | Source | Identifier |
|------|--------|------------|
| RNA-seq Data | European Nucleotide Archive (ENA), http://www.ebi.ac.uk/ena | ENA: ERP013883 |

**Experimental Models: Organisms/Strains**

| Name | Source | JAX: |
|------|--------|------|
| Mouse: R6/2: C57BL/6J | The Jackson Laboratory | 000664 |
| Mouse: R6/2: B6(Cg)-Tyr^c-2J/J | The Jackson Laboratory | 000058 |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents may be directed to and will be fulfilled by the Lead Contact, Tim J. Schulz (tim.schulz@dife.de). Animal strains used in this study are covered by MTAs prepared with the respective strain providers.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All procedures were approved by the ethics committee for animal welfare of the State Office of Environment, Health, and Consumer Protection (State of Brandenburg, Germany). Animals were housed in a controlled environment (20 ± 2 °C, 12/12 hr light/dark cycle), maintained on a SD (Sniff, Soest, Germany), or fed a HFD (45% energy from fat, D12451, Research Diets, New Brunswick, NJ, USA) for 1 and 10 days. Male mice were used for all experiments at the indicated ages. All following mouse strains were obtained from The Jackson Laboratory: C57BL/6J, B6(Cg)-Tyr c-2J/J (B6-albino), B6.Cg-Tg(Rosa26-Sor-EGFP)I1Able/J, B6.129S4-Pdgfra tm11(EGFP)Sor/J (Pdgfra-eGFP reporter), B6;FVB-Tg(Zfp423-EGFP)7Brsp/J (Zfp423-eGFP reporter), B6.SJL-Ptprc eGFP/BoyJ (CD45.1), B6.129(Cg)-Tg(Rosa26Sor-EGFP)Luc/J (mTmG-reporter), FVB.129S6(B6)-Gt(Rosa26Sor-EGFP)Luc/J (Rosa26-Luciferase reporter), B6.Cg-Tg(Prrx1-cre)1Cjt/J, C57BL/6-Tg(Pdgfra-cre)1Clc/J, B6;FVB-Tg(Adipoq-cre)1Evdr/J, B6.129-Tg(Cdh5-cre)1Spe/J, B6;129-Tg(Cdh5-cre)1Spe/J, B6.Cg-Tg(Vav1-icre)A2Kio/J, B6.Cg-Tg(Vav1-icre)1Evdr/J, B6.129-Cg-Tg(Cdh5-cre)1Spe/J, B6.Cg-Tg(Vav1-icre)1Evdr/J, B6.129-Tg(Cdh5-cre)1Spe/J, B6;FVB-Tg(Zfp423-EGFP)7Brsp/J (Zfp423-eGFP reporter), B6.SJL-Ptprc eGFP/BoyJ (CD45.1), B6.129(Cg)-Tg(Rosa26Sor-EGFP)Luc/J (mTmG-reporter), FVB.129S6(B6)-Gt(Rosa26Sor-EGFP)Luc/J (Rosa26-Luciferase reporter), B6.Cg-Tg(Prrx1-cre)1Cjt/J, C57BL/6-Tg(Pdgfra-cre)1Clc/J, B6;FVB-Tg(Adipoq-cre)1Evdr/J, B6.129-Tg(Cdh5-cre)1Spe/J, B6.Cg-Tg(Vav1-icre)A2Kio/J, B6.Cg-Tg(Tek-cre)1Ywa/J. The strain Dpp4tm1Nwa (Marguet et al., 2000; DPP4-KO) was provided from a colony maintained by Dr. Hua Fan from Charité University of Medicine, Berlin, Germany. Mouse strains expressing Cre-recombinase under promoter control of the hematopoietic (Vav1), endothelial (Cdh5 and Tek/Tie2), mesenchymal (Prx1 and PDGFRa) or mature adipocyte (AdipoQ) lineage markers were intercrossed with the mTmG-reporter mouse strain that constitutively expresses the membrane-bound red fluorescent protein tdTomato (from a loxP-flanked cDNA). Cre-mediated recombination leads to excision of the tdTomato-cassette and activates expression of green fluorescent protein instead. For transplantation experiments, Zfp423-eGFP reporter mice were either intercrossed with mTmG-reporter mouse strain (rep tdTom), or to AdipoQ-Cre mice and a lox-Stop-lox reporter strain expressing luciferase after Cre-mediated removal of the floxed Stop-cassette from the Rosa26-locus (rep Adluc). Freshly sorted primary murine cells were used throughout this study and isolated by FACS and cultured as described before (Schulz et al., 2011; Steenhuis et al., 2008). Cells were therefore not authenticated. For cultivation, a complex medium of 60% DMEM low glucose (Invitrogen) and 40% MCDB201 (Sigma) was supplemented with 100 U/mL penicillin and 1,000 U/mL streptomycin (Invitrogen), 2% FBS, 1 x insulin-transferrin-selenium (ITS) mix, 1 x linoleic acid conjugated to BSA, 1 mM dexamethasone, and 0.1 mM L-ascorbic acid 2-phosphate (all from Sigma) were added. Before use, growth factors were added to the medium: 10 ng/mL epidermal growth factor (PeproTech), 10 ng/mL leukemia inhibitory factor (MerckMilipore), 10 ng/mL platelet-derived growth factor BB (PeproTech), and 5 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich). The bFGF was added daily throughout the culture period except where stated otherwise. For adipogenic differentiation cells were induced for 48 hr after 3 days of expansion, followed by a differentiation period of 5 days. For adipogenic differentiation, induction medium (growth medium without growth factors) containing 5 μg/mL human insulin (Roche Applied Science), 50 μM indomethacin, 1 μM dexamethasone, 0.5 μM isobutylmethylxanthine, 1 nM 3,3',5-triiodo-L-thyronine (T3) (all from Sigma-Aldrich) was added for 48 hr, followed by further differentiation in growth medium without growth factors and the addition of T3 and insulin only. Oil Red O staining was performed by fixing cells with 4% HistoFix for 15 min at room temperature. For the preparation of Oil Red O working solution, a 0.5% stock solution in isopropanol was diluted with distilled water at a ratio of 3:2. The working solution was filtered and applied to fixed cells for at least one hour at room temperature. Cells were washed four times with tap

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: R6/2: B6.Cg-Tg(Rosa26Sor-EGFP)I1Able/J | The Jackson Laboratory | JAX: 007669 |
| Mouse: R6/2: B6;129S4-Pdgfra tm11(EGFP)Sor/J | The Jackson Laboratory | JAX: 007897 |
| Mouse: R6/2: B6;FVB-Tg(Zfp423-EGFP)7Brsp/J | The Jackson Laboratory | JAX: 019381 |
| Mouse: R6/2: B6;SJL-Ptprc eGFP/BoyJ | The Jackson Laboratory | JAX: 002014 |
| Mouse: R6/2: B6.129(Cg)-Gt(Rosa26Sor-EGFP)I1Able/J | The Jackson Laboratory | JAX: 007676 |
| Mouse: R6/2: FVB.129S6(B6)-Gt(Rosa26Sor-EGFP)Luc/J | The Jackson Laboratory | JAX: 005125 |
| Mouse: R6/2: B6.Cg-Tg(Prrx1-cre)1Cjt/J | The Jackson Laboratory | JAX: 005584 |
| Mouse: R6/2: C57BL/6-Tg(Pdgfra-cre)1Clc/J | The Jackson Laboratory | JAX: 013148 |
| Mouse: R6/2: B6;FVB-Tg(Adipoq-cre)1Evdr/J | The Jackson Laboratory | JAX: 010803 |
| Mouse: R6/2: B6;129-Tg(Cdh5-cre)1Spe/J | The Jackson Laboratory | JAX: 017956 |
| Mouse: R6/2: B6.Cg-Tg(Vav1-icre)A2Kio/J | The Jackson Laboratory | JAX: 008610 |
| Mouse: R6/2: B6.Cg-Tg(Tek-cre)1Ywa/J | The Jackson Laboratory | JAX: 008863 |
| Mouse: Dpp4 tm1Nwa | Marguet et al., 2000 | MGI ID: 2150161 |

Sequence-Based Reagents

qPCR Primer sets: please see Table S6, This paper N/A
Osteocalcin, and chondrogenic if it co-stained with Aggrecan. Alternatively, in a feeder cell-free assay, a single CD45-CD31-Sca1+ tdTomato+ cell freshly isolated from 8-weeks old male Rosa26-mTmG mice was FAC-sorted into each well. Cells were expanded for 10 days to sub-confluency with media changes every other day. After 10 days cells were fixed and stained with 1% Alcian-Blue staining (Sigma) for 30 min at room temperature. Cells were rinsed three times with 0.1 M HCl. To neutralize acidity a washing step with dH2O was conducted before microscopic analysis. For DPP4 in vitro experiments cell populations were differentiated with adipogenic or osteogenic assays as described above. Mouse recombinant DPP4 (250ng/mL; R&D Systems) or DPP-4 inhibitor Sitagliptin (100 μM; biomol) were added to differentiation cocktails from day 3 (adipogenic induction) during adipogenesis or day 0 during osteogenesis until the end of differentiation experiments. DPP4 secretion into cell culture media was determined by ELISA (Thermo Fisher). Either supernatant of freshly isolated tibia explants maintained in culture media for 24 hr or supernatant from cell populations following 10 days of adipogenic differentiation were used. CFU-F assay was conducted as follows: Freshly isolated cell populations were seeded in expansion media at 500 cells per 6-well plate. Medium was changed every other day. After 10 days cells were fixed and stained with Crystal Violet (Sigma). Colonies consisting of more than 20 cells were counted as CFU. At least 6 independent assays were performed per cell population. For total recovery rate experiments cell populations were seeded as described for the CFU-F assay. Analysis of fixed and Crystal Violet stained cell populations was conducted on day 7, 11, and 15 by quantification of total cell invasion area of well-plate surface using ImageJ software.

**METHOD DETAILS**

**Flow cytometry & cell sorting**

Flow cytometry and cell sorting were performed on a FACS Aria III cell sorter (BD Biosciences) and analyzed using FlowJo software (Tree Star). Soft-tissue free bones (tibia/femur) were crushed with bone scissors and incubated for 1 hr in a shaking water bath at 37°C in 10 mL of 20% FBS/PBS containing 0.5% type-2 collagenase (CellSystems). The suspension was filtered through a 70μm mesh to remove bone fragments and centrifuged at 1200 rpm for 5 min at 4°C. The pellet was re-suspended in ACK (Ammonium-Chloride-Potassium) lysing buffer to eliminate red blood cells and centrifuged again at 1200 rpm for 5 min at 4°C. The pellet was re-suspended in 100 μL sorting buffer (2% FBS/PBS) and stained with antibodies for at least 30 min at 4°C. The applied FACS antibodies can be found in the Key Resources Table. Living cells were gated for lack of PI (propidium iodide; 1:1,000 diluted stock solution: 1 μg/mL in water) fluorescence and accumulation of Calcein (1:1,000 dilution; stock of 1 mg in 215 μL DMSO). Compensation, fluorescence-minus-one control based gating, and FACS-isolation was conducted as described before using the antibody combinations as indicated in the respective figures and legends (Schulz et al., 2011).

**Single-cell clonal assays**

For the co-culture approach, a feeder layer of CD45+CD31+Pax+ cells was isolated from long bones of 8-weeks old male C57BL/6J mice and seeded in 100 μL of expansion medium at 750 cells per well of a 785-well plate. On the next day, a single CD45+CD31+Sca1+CD24+ tdTomato+ cell freshly isolated from 8-weeks old male Rosa26-mTmG mice was FAC-sorted into each well. Cells were expanded for 10 days to sub-confluency with media changes every other day. After 10 days, clonal expansion of a single cell was verified by fluorescence microscopy. Wells containing a readily detectable single colony of tdTomato+ cells were trypsinized, washed, and collected in 10 μL sorting medium. Five to ten cells (per condition) of each clone were directly FAC-sorted onto freshly prepared 96-well plate feeder layers of expanded CD45+CD31+Pax+ cells for adipogenic and osteogenic differentiation protocols, or onto a micromass culture for chondrogenic differentiation. At the end of the differentiation assays clones were analyzed for their differentiation capacity by immunocytochemistry. A tdTomato positive clone was considered adipogenic if it co-stained with Perilipin, osteogenic if it co-stained with Osteocalcin, and chondrogenic if it co-stained with Aggrecan. Alternatively, in a feeder cell-free assay, a single CD45+CD31+Sca1+CD24+ cell, freshly isolated from 8-weeks old male C57BL/6J mice, was FAC-sorted into a well of a 96-well plate without feeder cells. Single cells were expanded for 10 days with media changes every other day. After 10 days, colonies giving rise to colonies were re-seeded in a new 96-well plate and expanded until sub-confluency. Clones were then used for tri-differentiation assays. At the end of differentiation Oil Red O staining was conducted for adipogenesis and immunocytochemistry for osteogenesis (Osteocalcin) and chondrogenesis (Aggrecan). Images were acquired with a Keyence BZ-9000 (Biorevo) fluorescence microscope.

**Histology**

Isolated bones were cleaned from surrounding tissue and fixed/decalcified in Richard-Allan Scientific Cal-Rite fixative (Thermo Scientific), followed by paraffin embedding. Sections (3 μm) were used for immunohistological staining or H&E overview staining. For immunohistochemistry sections were de-paraffinized and re-hydrated in xylene and decreasing ethanol concentrations. Heat-mediated antigen retrieval was conducted by placing sections in blocking buffer (40 mM Tris and 1.2 mM EDTA in distilled water) in a
microwave for 5 min at 330 W. Slides were left for cooling and rinsed with water. Nearly dried samples were circled with a PAP pen (Kiszer) and incubated with blocking solution (1% BSA/PBS) for 60 min at room temperature. Primary antibodies, diluted in 1% BSA/PBS, were added and samples were incubated in a humidified chamber at 4°C overnight. Sections were washed with PBS three times. Secondary antibody and DAPI nucleus staining were applied for 10 min at room temperature in the dark. Samples were washed twice with PBS. To reduce auto-fluorescence 0.3% Sudan Black solution (in 70% EtOH) was applied for 20 min. Sections were washed three times and mounted with Fluoromount G (eBioscience, GER). Samples were stored at 4°C in the dark before evaluation via fluorescence microscopy. For the quantification of the different bone-resident populations, bone marrow regions of 0.05 mm² from bone sections were selected on fluorescence images. For immunocytochemistry, fixed cells in well plates were permeabilized with 0.1% Triton X-100 solution and blocked with 3% BSA in PBS. Antibodies were used as listed in the Key Resources Table. For nuclear staining specimen were treated with DAPI. Sections and cells were analyzed using a Keyence BZ-9000 (Bioerove) fluorescence microscope (for up to two fluorescences) or a Zeiss confocal laser scanning microscope (LSM) 700 (for three fluorescences).

### Sternal transplantation

Sorted cell populations (see Figure S2L) from luciferase-expressing rep<sub>AdiLuc</sub> or tdTomato-expressing rep<sub>tdTom</sub> mice were subcutaneously injected at 1.5 x 10⁶ cells in a 50% matrigel suspension into the sternal area of B6-albino mice. Eight weeks after transplantation, engrafted tissues were excised, fixed, and histologically analyzed. Mice injected with cells from rep<sub>Luc</sub> animals were additionally subjected to Luciferase imaging with an IVIS imaging system (Perkin Elmer) before sacrifice. To this end, animals were intraperitoneally injected with luciferin (150 mg/kg) and subsequently anesthetized. After 12 to 18 min, the animals were imaged. Image analysis was performed with Living Image 4.4 software (Xenogen).

### BrdU cell proliferation in vivo assay

For 24 hr experiments mice were i.p.-injected with a single dose of 100 mg BrdU/kg (Sigma Aldrich) diluted in sterile PBS. Mice receiving a SD or HFD for ten days were given BrdU via drinking water at a concentration of 0.5 mg/mL. Drinking water was refreshed every other day. For single-cell immunostaining approximately 2 x 10⁵ cells/mouse of each population of interest were double-sorted on glass coverslips pre-coated with a 5 µL drop of DMEM-low. Coverslips were incubated for 30 min, allowing cells to attach. Cells were fixed by gently adding 4% Histox for 10 min and washed three times for 3 min with PBS. Permeabilization solution (0.2% saponin/PBS) was applied for 6 min. Washing solution (0.02% saponin/PBS) for 5 min was followed by administering DNA-denaturation solution (2 M HCl in 0.02% saponin/PBS) for 20 min at 37°C. Cells were washed for 5 min and blocked for 30 min with 2% BSA in washing solution. Incubation with anti-BrdU antibody (Cedarlane) in blocking solution was done overnight at 4°C. On the next day, three times washing for 10 min and incubation with Alexa Fluor 488 donkey anti-rat (FisherScientific) and DAPI staining in blocking solution for 30 min at room temperature in the dark was performed. Cells were washed with washing solution three times and PBS once. Coverslips were mounted with Fluoromount G (eBioscience). Samples were stored at 4°C in the dark before evaluation via fluorescence microscopy (Keyence). The percentage of BrdU-positive cells within each population was calculated as compared to total numbers of DAPI-positive cells.

### Competitive hematopoietic reconstitution assay

Animals were given antibiotics via drinking water and an analgetic one week and two days, respectively, before experiments. C57BL/6J recipient mice were lethally irradiated with a single dose of 7.5 Gy. For reconstitution assays, 150 LT-LSK cells from donor mice expressing the CD45.1 allele were mixed with 1 x 10⁴ freshly isolated whole bone marrow supporting cells (collected by flushing the BM from long bones) from C57BL/6J mice (CD45.2) in addition to 1.5 x 10⁴ cells of one of the investigated populations isolated from rep<sub>atm</sub> mice, then injected in the medullary cavity of the tibia through the proximal articular surface. For the investigation of acute effects peripheral blood, tibia bone marrow, and tibia bones were analyzed by flow cytometry five weeks post irradiation. For long-term experiments blood was taken every four weeks and tibia bone marrow and tibia bones were analyzed 16 weeks post irradiation. Red blood cells were lysed, followed by antibody staining to distinguish between different blood cell populations (as listed in the Key Resources Table). For evaluation of donor chimerism the frequency of donor fraction (CD45.1⁺:CD45.2⁻) was calculated.

### Fracture model

Mice were given an analgetic (MediGel, ClearH₂O) starting two days prior to surgery. Anesthetized mice were injected with 1.5 x 10⁴ cells in a 50% matrigel suspension through the proximal articular surface of the tibia. A steel pin (diameter 0.35 mm) was inserted into the medullary cavity for stabilization and a fracture was induced with scissors 0.5 cm distal from the knee. At the indicated time point after fracture induction, tibiae were harvested for analyses. After removal of the pin from extracted tibiae, µCT analysis was conducted with LaTheta LCT-200 (Hitachi-Aloka) using manufacturer’s pre-defined parameters for isolated bone measurements. Alternatively, tibiae were fixed and decalcified followed by paraffin embedding and sectioning at 3 µm per slice. Samples were stained using SafraninO/Fast green and Movat Pentachrome. ImageJ software was used for computer-assisted histomorphometric analysis of fracture calluses. Six representative sections of each callus were analyzed for bone, fibrous, and cartilaginous tissue areas in a blinded manner. For DPP4 in vivo experiments mice received a daily dose of PBS, Diprotin A (5 mg/kg body weight; Sigma) or Sita-gliptin (10 mg/kg body weight; biomol) i.p. for 9 consecutive days. For fracture healing experiments application started two days before induction of injury/cell injection. Fracture healing was assessed one day after the last DPP4-inhibitor administration.
Capture, Library Preparation, and Sequencing of cell population
A total of 17,000 CD45^-CD31^-Sca1-Zfp423^+ 50,000 CD45^-CD31^-Sca1^+CD24^- 5,000 CD45^-CD31^-Sca1^+CD24^+ and 30,000 CD45^-CD31^-Sca1^+P^a^+ cells were FAC-sorted from bones of 4 mice (the 3 biological replicates were done on 3 different days), collected in a 1.5 mL Eppendorf tube containing 50 μL RLT Plus Buffer (QIAGEN) supplemented with 1% 2-Mercaptoethanol, immediately frozen in dry-ice and kept at −80°C. The time elapsed between mouse euthanasia and the termination of the FACS procedure was ~400 min. RNA extraction, reverse transcription and cDNA pre-amplification, Nextera XT libraries and sequencing of the cell populations was done as previously described and according to the manufacturer’s instructions. Briefly, RNA was extracted using the RNeasy Plus Micro Kit (QIAGEN), together with genomic DNA eliminator (QIAGEN). Reverse transcription and cDNA pre-amplification were performed using the SMARTer PCR cDNA Synthesis kit (Clontech) and the Advantage 2 PCR kit (Clontech). cDNA was harvested and quantified with the Bioanalyzer DNA High-Sensitivity kit (Agilent Technologies). Libraries were prepared using the Nextera XT DNA Sample Preparation Kit and the Nextera Index Kit (Illumina). Multiplexed libraries were pooled, and paired-end 100-bp sequencing was performed on one flow–cell (two lanes) of an Illumina HiSeq 2500.

RNA-seq data processing and analysis
Sequencing data were aligned to the Mus musculus genome (Ensembl version 38.82) using GSNAP (version 2014-10-07) with default parameters. HTseq-count was used to count the number of reads mapped to each gene (default options). Almost all libraries showed good quality, with sizes ranging between 2-3.5x10^7 read counts and a fraction of reads mapped to exons greater than 75% (Figure S7). One library yielded less than 300 reads and was excluded from downstream analysis. The data was normalized for sequencing depth using size factors. The union of the top 1,000 genes expressed in each library was selected, which resulted in a list of 2,120 genes. Principal component analysis was carried out on the standardized log10-transformed normalized counts (after adding a pseudo-count of 1 to avoid infinities). Hierarchical clustering analysis was performed using Euclidean distances with Ward’s method on the same dataset. Differentially expressed genes between groups of libraries were identified by using the bioconductor R-package DESeq2 library at a FDR of 0.1. Genes that were not detected in any library were removed prior to the analysis and possibly confounding factors were taken into account (i.e., the animal each sample was taken from). RNA-seq data was statistically analyzed using the R-statistical package and Paleontological Statistics (PAST, version 3.10, http://folk.uio.no/ohammer/past/, accessed December 2015). For DE analyses, gene expression was compared between all investigated cell populations. A p-value of < 0.05 was used as a cut-off for differentially expressed genes. Heat-maps contain representative top-regulated genes, which were further divided by known cell type specific functions as previously described in the literature and unknown novel marker genes.

Gene expression analysis
Total RNA isolation and gene expression analysis was conducted using standard methods as described before (Schulz et al., 2011) using column-based RNA-isolation, reverse transcription for cDNA synthesis, and SYBR green-based detection during quantified real-time PCR. Primer sequences were used as noted in the Key Resources Table.

QUANTIFICATION AND STATISTICAL ANALYSES
All data are presented as mean ± standard error of the mean (SEM). The sample size for each experiment and the replicate number of experiments are included in the figure legends. Statistical significance was defined as p < 0.05. Statistical analyses were performed using unpaired, two-tailed Student’s t test or Mann-Whitney-U-test where applicable for comparison between two groups, and an ANOVA test was used for experiments involving more than two groups (GraphPad Prism; version 6.04).

DATA AND SOFTWARE AVAILABILITY
Data Resources
The accession number for the gene expression data generated by RNA-seq data reported in this study is ENA: ERP013883 (http://www.ebi.ac.uk/ena).
Supplemental Information

Adipocyte Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based Hematopoietic and Bone Regeneration

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Supplemental Figure 3

(A) OPP CD45-CD31+Sca1+CD24+ APC PreAd

(B) CD45-CD31+Sca1+CD24+ APC PreAd

tdTomato Perilipin DAPI

(C) tdTomato CD45 DAPI

(D) Merge: tdTomato Zfp423-eGFP DAPI

(E) Merge: tdTomato Perilipin DAPI

(F) CD45-CD31+Sca1+ cells

(G) Prx1-Cre

(H) Bone iWAT BAT Muscle

(-Rosi) +Rosi

(I) Pparg Gene exp (% of bone w/o Rosi)

(J) Cebpa Gene exp (% of bone w/o Rosi)

(K) Ucp1 Gene exp (% of bone w/o Rosi)

(L) Cidea Gene exp (% of bone w/o Rosi)

(M) UCP1 Western Blot
Supplemental Figure 5

A) BM Lymphoid

B) BM Myeloid

C) Blood Chimerism

D) Spleen LSK

E) Bone marrow LSK

F) LT-LSK cells

G) ST-LSK cells

H) Blood chimerism

I) Blood lymphoid

J) Blood myeloid

K) No cells, OPC, CD45-CD31- Sca1-CD24+, APC

L) Frequency of CD45-CD31-Sca1-CD24+ cells (% of alive)
| Differentiation potential | Single-cell co-culture assay (with feeders) # of clones | Single-cell assay (without feeders) # of clones |
|---------------------------|--------------------------------------------------------|------------------------------------------------|
| Adipogenic/Osteogenic/Chondrogenic | 64 (94.12%) | 45 (83.33%) |
| Adipogenic/Osteogenic | 2 (2.94%) | 1 (1.85%) |
| Adipogenic/Chondrogenic | 1 (1.47%) | - |
| Osteogenic/Chondrogenic | - | - |
| Adipogenic only | 1 (1.47%) | 4 (7.42%) |
| Osteogenic only | - | 3 (5.55%) |
| Chondrogenic only | - | - |
| none | - | 1 (1.85%) |
| **Total # of clones** | **68** | **54** |
| Population (% of viable cells) | Long bones | Sternum | Thoracic vertebra | Caudal vertebra | Calvarium |
|-------------------------------|------------|---------|------------------|----------------|-----------|
| Multi-potent stem cell (CD45-CD31-Sca1+CD24+) | 0.03967\(^c\) ± 0.0043 | 0.032\(^c\) ± 0.003 | 0.23 ± 0.065 | 0.03967\(^c\) ± 0.006 | 0.04767\(^c\) ± 0.004 |
| Osteochondrogenic progenitor cell (OPC) | 0.51\(^c,d,e\) ± 0.038 | 0.47\(^c,d,e\) ± 0.01 | 1.077\(^d,e\) ± 0.23 | 0.6133\(^a\) ± 0.024 | 0.78 ± 0.023 |
| Adipogenic progenitor cell (APC) | 0.18\(^c\) ± 0.015 | 0.07333\(^c\) ± 0.023 | 0.6267 ± 0.08 | 0.1467\(^c\) ± 0.007 | 0.109\(^c\) ± 0.007 |
| Pre-adipocyte (preAd) | 0.1333\(^b,c,d,e\) ± 0.033 | 0.09133\(^c,d\) ± 0.019 | 0.02567\(^e\) ± 0.003 | 0.04733 ± 0.01 | 0.07333 ± 0.003 |
| Transplant fate | Cell population | OPC | CD45-CD31- + Sca1+CD24+ | APC | predAd |
|-----------------|-----------------|-----|--------------------------|-----|--------|
| Adipogenic      | -               | 2   | 13                       | 5   |        |
| Osteochondrogenic | 9              | 2   | -                        | -   | -      |
| Adipog. & osteochondr. | -            | 3   | -                        | -   | -      |
| No engraftment  | 2               | 3   | 3                        | 1   |        |
| n (animals)     | 11              | 10  | 16                       | 6   |        |
| Population                          | Immunophenotypes/Marker phenotypes | Adipogenic potential                  | Osteogenic potential                  | Chondrogenic potential |
|-----------------------------------|------------------------------------|---------------------------------------|---------------------------------------|------------------------|
| Multi-potent stem cell-like population | CD45-CD31-Sca1+Pα+CD24+ Zfp423- | In vitro, sternal transplant, fracture model, irradiation model | In vitro, sternal transplant, fracture model | In vitro, sternal transplant, fracture model |
| Osteochondrogenic progenitor cell (OPC) | CD45-CD31- Sca1-Pα+CD24+ Zfp423- | No adipogenesis                       | In vitro, sternal transplant, fracture model | In vitro, sternal transplant, fracture model |
| Adipogenic progenitor cell (APC)   | CD45-CD31- Sca1-Pα+CD24+ Zfp423- | In vitro, sternal transplant, fracture model, irradiation model | No osteogenesis                      | No chondrogenesis |
| Pre-adipocyte (preAd)             | CD45-CD31-Sca1- CD24 Zfp423+      | In vitro, sternal transplant, fracture model, irradiation model | No osteogenesis                      | No chondrogenesis |
| Gene   | Sequence (5’→3’)                      | Species | Accession No. |
|--------|---------------------------------------|---------|---------------|
| Cebpa  | Fwd: AGTCGGTGAGACAAGACAGC             | mouse   | NM_007678     |
|        | Rev: TCACTGGTCAACTCCAGCA              |         |               |
| Cidea  | Fwd: ATCAAACTGGCCTGGTTACG             | mouse   | NM_007702     |
|        | Rev: TACTACCCGCTTCACCTTCT             |         |               |
| Dpp4   | Fwd: CGGTATCATTTAGTAAAGAGGCAA        | mouse   | NM_010074     |
|        | Rev: GTAGAGTGAGAGGGGCAGAC             |         |               |
| Fabp4  | Fwd: GATGCCTTTTGGGAAACCT             | mouse   | NM_024406     |
|        | Rev: CTGTGTCTGGCGGTGATT              |         |               |
| Lep    | Fwd: CCTCATCAAGAGCATGCACC            | mouse   | NM_008493     |
|        | Rev: TCTCCAGGTCATTGGCTATCTG          |         |               |
| Ppia   | Fwd: CAAATGCTGGACCAAAACAA            | mouse   | NM_008907     |
|        | Rev: AAGACCACATGCTTGCCAT             |         |               |
| Pparg  | Fwd: CTCCAAGAATACCAAGTGCGA           | mouse   | NM_011146     |
|        | Rev: GCCTGATGTCTTATCCCAAA            |         |               |
| Osx/Sp7| Fwd: TCCTCGGTCTCTCCATCTG             | mouse   | NM_130458     |
|        | Rev: GGACTGGAGCCATAGTGAGC            |         |               |
| Runx2  | Fwd: TTCAACGATCTGAGATTGTGGG          | mouse   | NM_001146038  |
|        | Rev: GGATGAGGAATGCGCCCTA             |         |               |
| Ucp1   | Fwd: CAAATCAGCTTTGCCCTACTC           | mouse   | NM_009463     |
|        | Rev: TAAGCCGCTGAGATCTTGT             |         |               |
**Supplemental Figure legends**

**Supplemental Figure 1, related to Figure 1. Common Progenitor Cell Markers Identify Functionally Distinct Bone Marrow-resident Populations of Mesenchymal Cells.** (A) Representative dot plots showing FACS staining for CD31/CD45 (x-axis) and Sca1 (y-axis) of live stroma cells derived from bone, subcutaneous (inguinal) white adipose tissue (iWAT), brown adipose tissue (BAT), and muscle. CD45+CD31-Sca1+ cell populations of each tissue are indicated in black circles. (B) FACS-analysis of viable cells from 2-month-old male Pα-EGFP reporter mice for expression of GFP followed by Sca1 and CD45/CD31 expression analysis within GFP+ cells. (C) Adipogenic (Oil Red O) and osteogenic (Alizarin Red S) differentiation assays of Sca1+Pα- and Sca1+Pα+ populations. (D, E) CFU-F (n=6/cell population, D) and total recovery rate (n=3/cell population, E) assays of the indicated bone populations. (F, G) Flow cytometric dot plot analysis (F) and adipogenic (Oil Red O), osteogenic (Alizarin Red S), and chondrogenic (Alcian Blue) differentiation potentials (G) of CD45+CD31-Sca1+Pα- cells separated into CD24+ (upper panels) and CD24- (lower panels) cell populations. (H) Dot plot analysis of the bone resident CD45+CD31+Sca1+Pα+ cell population by separation into CD24+ and CD24- cells by FACS. (I) Representative image of clonal analysis of single CD45+CD31+Sca1+CD24+ cells grown without feeder layers by staining of clone 22-derived cells for Oil Red O-accumulation, Osteocalcin or Aggrecan to show adipogenic, osteogenic, and chondrogenic differentiation potentials, respectively. (J) FACS-analysis of CD45+CD31+ cells from 2-months old male Zfp423-EGFP reporter mice for expression of Sca1 and GFP. (K) GFP-fluorescence was assessed in cultured CD45+CD31+Sca1- (left) and Zfp423+ (right) cells before (top panels) and after (bottom panels) adipogenic differentiation. (L, M) Analysis (L) and significant correlation (M) of average fluorescence intensities (AvInt) in individual cells (n=72) after immunofluorescence co-staining for Sca1 (red fluorescence) and Zfp423-EGFP (green fluorescence) in the CD45+CD31+Sca1+ population at day 5 of adipogenic differentiation (green arrow: Sca1+Zfp423-; orange arrow: Sca1+Zfp423+). (N) FACS-analysis of cultured Sca1+ cells at day 3 (d3), 5 (d5), and 8 (d8) of adipogenesis showing frequencies of Sca1+Zfp423, Sca1+Zfp423+, and Sca1+Zfp423- cell populations (n=3). All values are shown as mean ± SEM. Scale bars, 30µm.

**Supplemental Figure 2, related to Figure 1. Distinct Anatomical Localization of Bone-resident Progenitor Cell Populations.** (A) To left: IF of a representative distal femur from Pα-EGFP reporter mice stained for EGFP (red, due to secondary antibody), Sca1 (green), CD24 (white), and DAPI (blue). To right: Metaphyseal (upper right panels) and diaphyseal (lower right panels) areas were enlarged to indicate micro-anatomical localizations of osteogenic Sca1+Pα- (blue arrows), multipotent Sca1+CD24-Pα+ (red arrows), and adipogenic Sca1+Pα+CD24- (orange arrows) within the endosteum, <40 µm, or >40 µm from the endosteum (areas indicated by broken white lines: b – bone; gp – growth plate; mp – metaphysis; dp – diaphysis). Scale bars, 50µm. (B) Quantification of osteogenic Sca1+Pα+ multipotent Sca1+Pα+CD24+ and adipogenic Sca1+Pα+CD24- cells in metaphysis or diaphysis of bones derived from Pα-EGFP mice using images as shown in panel A. (C) Quantification osteogenic Sca1+Pα- multipotent Sca1+Pα-CD24+ and adipogenic Sca1+Pα-CD24- cells localizing at the endosteum, or to areas <40 µm from the endosteum, and to areas >40 µm from the endosteal layer using images as shown in panel A. (D) Representative IF images of Sca1+ cell distribution in Pα-EGFP mice. Pα-GFP+ cells (red fluorescence due to secondary antibody) either reside in bone-linings, e.g. the endosteum (blue arrows), or associate to CD31+ (green fluorescence) blood vessels distributed within the bone marrow (yellow arrows). Scale bar, 10µm. (E) Quantifications of bone marrow-localized Pα-GFP+ cells associated to blood vessels with diameters, e.g. smaller or larger than 10 µm. (F, G) IF analysis of bones from Zfp423-EGFP mice: Zfp423+ cells (red fluorescence for GFP protein detection) either co-localizing with CD31+ (green fluorescence, F) or Perilipin (green fluorescence, G). Yellow arrows indicate blood vessel associated, undifferentiated Zfp423+ cells while only mature Zfp423+ bone marrow adipocytes co-stain with Perilipin. Scale bars, 10µm. (H) Quantification of Zfp423+ preAd distribution in bones from the Zfp423-EGFP reporter mouse strain in metaphysis or diaphysis. (I) Analysis of adipogenic potential (Oil Red O staining) of CD45+CD31+Sca1+ populations isolated from different bone compartments by FACS. Scale bar, 30µm. (J) Transgene alleles of the repAdluc reporter mouse strain: The Zfp423-EGFP reporter mouse strain was crossed to a strain expressing Cre-recombinase under control of the Adiponectin promoter (Adipoq-Cre) and a constitutive Luciferase (Luc)-reporter where the Luc-encoding cDNA is suppressed by a loxP-flanked Stop-signal. Thus, in this reporter strain only mature adipocytes expressing the adipogenic marker Adiponectin undergo Cre-mediated recombination. This leads to the excision of the loxP-flanked stop-cassette, activating the expression of Luciferase that can be detected by in vivo imaging techniques (reporter strain is referred to as repAdluc throughout the main text). (K) Transgene alleles of the repEtdTom reporter mouse strain: The Zfp423-EGFP reporter was crossed to an mTmG-reporter mouse strain without presence of a Cre-transgene. Thus, the cells maintained constitutive red fluorescence and can be detected by immunofluorescence for tdTomato or in vivo imaging (reporter strain is referred to as repEtdTom throughout the main text). (L) FACS-gating strategy for the isolation of the four investigated bone populations from both reporter mouse strains for subsequent in vivo transplantation assays. All results are shown as mean ± SEM (n=14-24 bone marrow sections were analyzed from n=4 mice per reporter strain; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).
Supplemental Figure 3, related to Figures 1 and 2. MAT Derives From a Multipotent Population with Stem Cell-like Potential and Resembles WAT rather than a BAT. (A, B) IF of sternal-grown tissues derived from transplanted cell populations of rep^Tom reporter mice (Red: tdTomato; Green: Perilipin (A) or Zfp423-EGFP (B); Blue: DAPI). Scale bars, 20µm. (C) IF of CD45^CD31 Sca1^+ Ptα^+ OPC derived tissue (red) with donor-derived bone-like structures containing host-derived CD45-expressing cells (green, white arrows). Scale bar, 20µm. (D) IF of tdTomato (red) and Zfp423-reporter driven GFP (green, top panels) or Perilipin (green, bottom panels) expression (merged with blue DAPI stain) after intratibial injection of donor cells derived from mTmG (no Cre-expression)-Zfp423-EGFP (rep^Tom) double-transgenic mice. Red arrows indicate tdTomato⁺, e.g. donor-derived, cells, white arrows indicate double-positive cells expressing the Zfp423-driver, e.g. cells committed to the adipogenic lineage. Scale bar, 10µm. (E) FACS-analysis of transplants of initially CD45^CD31 Sca1^+CD24⁺ cells identified by tdTomato-expression show that these cells give rise to the CD45^CD31 Sca1^+CD24⁺ population within the transplant. (F) FACS-analysis of bones derived from Ptα-EGFP mice showing a full overlap of Ptα-antibody and EGFP labeled CD45^CD31 Sca1⁺ cell populations in BM of injected and contralateral tibiae of irradiated mice 5 weeks after irradiation/transplantation. Donor-derived bone marrow (BM) lymphoid (A) and myeloid (B) cell populations in BM of injected and contralateral tibiae of irradiated mice 5 weeks after irradiation/transplantation. (C) Blood chimerism and (D) splenic LSKs 5 weeks post transplantation. (E) Donor-derived LSK, (F) LT-LSK, and (G) ST-LSK hematopoietic stem cells in injected tibiae 16 weeks after irradiation/transplantation. p<0.05: a vs. no cells; b vs. CD45^CD31 Sca1 Ptα; c vs. CD45^CD31 Sca1^+ ; d vs. BM Ptα; e vs. CD45^CD31 Sca1^+CD24⁺; f vs. CD45^CD31 Sca1^+CD24⁻; g vs. CD45^CD31 Sca1^+CD24⁻; h vs. CD45^CD31 Sca1^+CD24⁻. Blood chimerism showing donor-derived blood cells at 4, 8, 12, and 16 weeks post transplantation. (I) Donor-derived bone marrow (BM) lymphoid and (J) myeloid cell populations in blood of irradiated mice 8 and 16 weeks after irradiation/transplantation. (K) FACS analysis of tibiae to identify transplanted cells from rep^Tom double-transgenic mice 16 weeks after irradiation/transplantation: CD45^CD31^+Ptα⁻ cells (red squares, top row) were gated for expression of GFP (green squares: Zfp423-EGFP reporter; bottom row), indicating that transplanted cells show long-term presence. (L) The effect of irradiation (7.5 Gy) on the tibia-resident CD45^CD31 Sca1^+CD24⁺ cell population. All results are displayed as mean ± SEM (*p<0.05; **p<0.01).
(red) in the multipotent CD45-CD31+Sca1+CD24-, adipogenic CD45-CD31+Sca1+CD24- (APCs), and CD45-CD31+Sca1-Zfp423+ (preAd) subpopulations that were not observed in transplants from osteogenic CD45-CD31+Sca1+Pa+ cells. Scale bars indicate 20 µm. (D) IF showing the contribution of transplanted multipotent CD45-CD31+Sca1+CD24- (upper panels) and osteogenic CD45-CD31+Sca1+Pa+ (OPC, lower panels) cell populations to osteochondrogenic structures in the fractured tibiae that were not observed in adipogenic cell transplants (Red: tdTomato; Blue: DAPI; right panels indicate merge of IF and light microscopic images). Scale bar, 20 µm. (E) IF showing the contribution of transplanted multipotent CD45-CD31+Sca1+CD24- (upper panels) and osteogenic CD45-CD31+Sca1+Pa+ (OPC, lower panels) cell populations to endosteal bone linings in the fractured tibiae (Red: tdTomato; Blue: DAPI; dotted lines indicate areas of compact bone as seen in right-side panels of merged IF and light microscopic images). Scale bar, 10 µm. (F, G) IF co-staining of tdTomato+ cells (red fluorescence) with Osteocalcin (F) or Aggrecan (G) to show osteogenic and chondrogenic differentiation fates of transplanted multipotent CD45-CD31+Sca1+CD24+ (upper panels) osteogenic CD45-CD31+Sca1+Pa+ (lower panels) cell populations. No co-staining detected in adipogenesis-committed populations, e.g. APCs and preAds (not shown). Scale bar, 10 µm.

Supplemental Figure 7, related to Figures 6 and 7. Delayed Fracture Healing Through Adipogenic Cells is Reversed by DPP4 Inhibition. (A) Results of RNA-Seq samples regarding read counts and (B) fraction of reads mapped to exons are displayed. (C) FACS analysis of DPP4/CD26 surface marker expression in CD45+CD31+ populations and CD45-CD31+Sca1+CD24-, CD45-CD31+Sca1+Pa+ (OPC), CD45-CD31+Sca1+CD24- (APC) and CD45-CD31+Sca1-Zfp423+ (preAd) populations of 2-months old male mice. (D) DPP4 release into the culture medium by OPCs, multipotent CD45-CD31+Sca1+CD24-, and CD45-CD31+Sca1+CD24+ cell populations that underwent in vitro adipogenic differentiation (n=3). (E) Oil Red O staining and (F) mRNA expression levels of Pparg and Lep in multipotent CD45-CD31+Sca1+CD24- and APCs (CD45-CD31+Sca1+CD24-) either treated with PBS (control; white bars) or Sitagliptin (100 µM, black bars) during adipogenic differentiation (n=6 from two independent experiments). (G) Alizarin Red S staining and quantification, and (H) mRNA expression levels of Osx and Runx2 in multipotent CD45-CD31+Sca1+CD24+ and OPCs (CD45-CD31+Sca1+Pa+) either treated with PBS (control; white bars) or recombinant mouse rDPP4 (250 ng/mL; blue bars) during osteogenic differentiation (n=3). (I) Oil Red O staining and (J) mRNA expression levels of Pparg and Lep in multipotent CD45-CD31+Sca1+CD24+ and APCs (CD45-CD31+Sca1+CD24- ) cells either treated with PBS (control; white bars) or rDPP4 (250 ng/mL; blue bars) during adipogenic differentiation (n=3). (K) Histomorphometric (top panels) analysis and corresponding µCT images (bottom panels) of the fracture callus and (L) quantification of mineralized (BV/TV) and fibrotic areas (FV/Tv) of mice either treated with PBS, Diprotin A, or Sitagliptin for 9 days (n=6-7). Results are shown as mean ± SEM (*p<0.05; **p<0.01). Scale bars, 30 µm.

Supplemental Table 1, related to Figure 1. Summary of differentiation potential analysis of 68 clones (left column: with feeder layer) and 54 clones (right column: without feeders) derived from single primary CD45-CD31+Sca1+CD24+ cells of bone. See also Figure 1E for a representative image depicting analysis of sample clone 19 from the feeder-based assay and Figure S1F for sample clone 22 of the feeder cell-free clonal assay.

Supplemental Table 2, related to Figure 1. Frequency analyses of the four investigated cell populations in different bone compartments by FACS. All results are displayed as mean ± SEM (n=3; *p<0.05: a vs. long bones; b vs. sternum; c vs. thoracic spine; d vs. caudal spine; e vs. calvarium).

Supplemental Table 3, related to Figure 1. Summary of sternal transplantation experiments of the four investigated cell populations including numbers of transplanted animals and respective differentiation fates as determined by histological analysis and engraftment efficiency with animals were no transplant was found.

Supplemental Table 4, related to Figure 1. Summary of the four investigated cell populations with phenotypic marker expression and differentiation potential performances during in vitro and in vivo experiments. Column for immunophenotypes/ marker phenotypes: Markers that are required to define and isolate the respective populations by flow cytometry are labeled in bold.

Supplemental Table 5, related to Figure 6. List of genes of RNA-seq displayed in heat maps of Figure 6 as well as expression patterns of genes reported for osteochondrogenic skeletal stem cells (Chan et al., 2015; Worthley et al., 2015).

Supplemental Table 6, related to Figures 7, S3, S4, and S7. Primer sets used in this study.