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# The transcriptional profile of adipose-derived stromal cells (ASC) mirrors the whitening of adipose tissue with age

Ilaria Scambi [a], Daniele Peroni [b], Alice Nodari [a], Flavia Merigo [a], Donatella Benati [a], Federico Boschi [c], Silvia Mannucci [a], Andrea Frontini [d], Silvia Visonà [e], Andrea Sbarbati [a], Mauro Krampera [f], Mirco Galiè [a,*]

[a] Department Neuroscience, Biomedicine and Movement, Sec. Anatomy and Histology, Universitity of Verona, Verona, Italy
[b] Laboratory of Translational Genomics, Centre for Integrative Biology, University of Trento, Trento, Italy
[c] Department of Computer Science, University of Verona, Strada Le Grazie 15, 37134 Verona, Italy
[d] Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy
[e] Department of Public Health Experimental and Forensic Medicine, University of Pavia, Pavia, Italy
[f] Department of Medicine, Section Hematology, Stem Cell Research Laboratory, University of Verona, Italy

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ABSTRACT

Multipotent stem cells persist within the stromal vascular fraction (SVF) of adipose tissue during adulthood. These cells, commonly referred to as adipose-derived stromal cells (ASC), have been extensively investigated over the past years as a promising therapeutic tool based on their regenerative and immunomodulatory properties. However, how ASC might mirror the age-related alteration of the fat they reside in remains unclear. Herein, we show that inguinal adipose tissue in mice turns from brown/beige- to white-like with age and resident ASC readily mirror these changes both at mRNA and microRNA transcriptional level. Mechanistically, our data suggest that these brown/age-related changes in ASC transcription rely on changes in the activity of E2F1 and NFkB transcription factors.

## 1. Introduction

Adipose tissue is traditionally categorised into white adipose tissue (WAT) and brown adipose tissue (BAT), both displaying unique features in terms of morphology and function (Peirce et al., 2014). A third category of adipose tissue, named beige, has recently been identified. This consists of brown-like cells interspersed within classic WAT depots and so these cells are also called "brite (brown in white)" adipocytes (Petrovic et al., 2010; Wu et al., 2012). Beige adipocytes may reversibly transition between brown- and white-like states in response to specific stimuli such as cold exposure, sympathetic stimulation and exercise (Ikeda et al., 2018).

Throughout postnatal life, WAT, BAT and beige adipose tissue undergo global remodelling. While WAT increases in mass during adulthood (Brookheart et al., 2009; Samuel and Shulman, 2012), BAT disappears after infancy, occasionally remaining within specific anatomical sites in adults such as the supraclavicular, subscapular and cervical regions. (Cypess et al., 2009) Brite adipocytes interspersed within subcutaneous WAT diminishes during adulthood and disappear by around 12 months of age in unstimulated rodents (Ikeda et al., 2018; Rogers et al., 2012).

Multipotent cells with adipogenic plasticity persist within the stromal vascular fraction (SVF) of fat depots during adulthood (Cawthorn et al., 2012). These cells are commonly referred to as adipose-derived stromal cells (ASC) (Pittenger et al., 1999). ASC represent the adipose-resident pool of a heterogeneous lineage of stem cells widely distributed throughout the body, so-called mesenchymal stem cells (MSCs), the potency of which may extend beyond mesenchymal phenotypes as they are able to differentiate into derivatives of all three germ layers (Jiang et al., 2002).

ASC take on multiple aspects of the different "shades" of fat they derive from, as well as their pathophysiological states (Berry et al., 2017; Macotela et al., 2012; Tchkonia et al., 2006; Timmons et al., 2007; Wu et al., 2012). However, little is known about how it may mirror the age-related changes of the fat depot they reside in. Functional network analysis based on gene expression have proven useful for identifying

* Correspondence to: Dept. Neuriscience, Biomedicine and Movement – Sec. Anatomy and HIstology, University of Verona, Via Le Grazie 8, 37134 Verona, Italy.
*E-mail address:* mirco.galie@univr.it (M. Galiè).

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different stem cell states (Kumar et al., 2014; Müller et al., 2008; Ramalho-Santos et al., 2002) and discriminating pathological changes in stem cell identity (Ben-Porath et al., 2008; Wong et al., 2008).

We show herein that the transcriptional profile of subcutaneous fat depot-resident ASC reflects the progressive "whitening" this depot incurs in with age. Specifically, we show that ASC explanted from the inguinal fat pad of 1-month-old mice are enriched in genes related to brown/beige-like transcriptional profile compared to 12-months-old mice, both in terms of mRNA and microRNA transcripts. Also, we show that transcriptional differences are ontologically related to the transcriptional activity of NFkB and E2F1 transcription factors, thus suggesting these two factors as master regulators of the beige-to-white conversion with age.

## 2. Material and methods

### 2.1. Animals

FVB mice were housed in groups of four or five with food and water available ad libitum in light- and temperature-controlled environments. The investigation complied with national legislation about the care and use of laboratory animals. Animal experiments were in accordance with the Amsterdam Protocol on animal protection and welfare and conducted according to the guidelines of Federation of European Laboratory Animal Science Associations (FELASA). Experimental protocol was approved by the "Interdepartmental Centre of Experimental Research Service" (CIRSAL) of the University of Verona.

### 2.2. Surgical excision and characterization of murine inguinal fat pad

The inguinal fat pad was excised from FVB mice aged 1(n = 3), 3(n = 3), 6(n = 3), or 12(n = 3) months and embedded in paraffin. 5 areas for each hematoxylin-stained section were analyzed in light microscopy, and the number of adipocytes was quantified in each area by counting nuclei with ImageJ. Adipocyte mean sizes were quantified by dividing the areas under analysis by the number of adipocytes counted in that area.

### 2.3. Isolation of adipose derived MSCs

The inguinal fat pads of FVB mice aged 1 (n = 8) and 12 (n = 8) months were surgically excised, weighed and processed to isolate the adipose-derived stem cells, according to standard protocols (Peroni et al., 2008). Briefly, the extracellular matrix was digested with collagenase (1 mg/ml) and centrifuged to obtain a high-density pellet, the stromal vascular fraction (SVF). After centrifugation, the SVF cell number was determined using light microscopy, and the cells were plated at a concentration of $1 \times 10^5$ cells/cm$^2$ using DMEM medium (with high glucose concentration, GLUTAMAX I™, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin). After 2–3 weeks of culture, a homogeneous cell population was obtained. The cells were identified as MSCs on the basis of their immunophenotype. Specifically, the positivity of CD106 (VCAM1), CD73, CD29, CD44, CD90 and the lack of hematopoietic (antiCD45, CD14, CD11c, CD123 and CD34 monoclonal antibodies) and endothelial cell markers (with CD31 monoclonal antibody) were assessed by means of cytofluorimetric analysis. Although the relatively long period of culture may introduce some alterations in ASC biology, it allows to get rid the transitory effects of microenvironment and to focus on the stable, intrinsic impact of aging of the ASC biology.

### 2.4. Microarray analysis

Whole-genome microarray analysis of MSC from differently aged mice was performed using the NimbleGen Gene Expression system. Briefly, total RNA was isolated from samples using the Qiagen RNeasy kit (Qiagen), following the manufacturer's instructions. RNA was used for cDNA synthesis followed by labeling of the cDNA with Cy3. The labeled cDNA samples were hybridized to *Mus musculus* 12×135K Array (Roche NimbleGen) which represents 44,170 mouse genes. The single color NimbleGen arrays were scanned with GenePix 4400 A Microarray Scanner. The data were extracted from scanned images using NimbleScan software and the Robust Multichip Average (RMA) algorithm was used to generate gene expression values. Hybridization, scanning and normalization of the data were performed as service by the Functional Genomic Center of the University of Verona (Verona, Italy). Raw and processed data are available at Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/) under the Accession no. GSE25069.

### 2.5. Transmission electron microscopy

Samples were fixed in 2% glutaraldehyde in Sorensen's buffer (pH 7.4) for 2 h, post-fixed in 1% osmium tetroxide in an aqueous solution for 2 h, dehydrated in graded concentrations of acetone, embedded in Epon-Araldite and cut with an Ultracut E Ultra-microtome (Reichert, Wien, Austria). At the end of the dehydrating process, samples were positioned in a multi-well grid for electron microscopy and observed using a TEM Morgagni 268D (FEI Philips). Quantification of mitochondrial size was performed using ImageJ software on 20 images of randomly selected fields taken at the same magnification.

### 2.6. MicroRNA profiling

Total RNA was isolated from each sample using the TRIzol reagent (Invitrogen), followed by further purification using a RNeasy kit (Qiagen, Hamburg, Germany) with slight modifications to preserve miRNAs. Briefly, 350 μl of buffer RLT and 3.5 × the volume of 100% ethanol were added to 50 μl of the RNA sample, then the mixture was pipetted into an RNeasy Mini spin column. After centrifugation, the column was washed twice with 500 μl buffer RPE and RNA was eluted with 30 μl RNase-free water.

Samples were then delivered to Exiqon (Vedbaek, Denmark) for labelling and hybridization using a Exiqon miRCURY™ LNA array (version 9.2; http://www.exiqon.com). This array contains 2000 capture probes covering all human, mouse and rat miRNAs, annotated in miRBase 9.2. Data are available in the Gene Expression Omnibus (GEO) database (GSE25685, SubSeries GSE25679).

### 2.7. Constructs and transfection

E2F1-Luc cells and NFkB-Luc cells were generated by lentivital infection with pGreenFire1_E2F1RE and pHAGE-NFkB-Luc-TdTomato, respectively. pGreenFire1_E2F1RE was a gift from Simone Di Giovanni (Addgene plasmid # 112248; http://n2t.net/addgene:112248; RRID: Addgene_112248) and encodes *Luciferase* under E2F1 responsive element sequence. pHAGE NFKB-TA-LUC-UBC-dTomato was a gift from Darrell Kotton (Addgene plasmid # 49335; http://n2t.net/addgene:49335; RRID:Addgene_49335) and encodes for *Luciferase* under the NFkB consensus binding sequence and *TdTomato* under UbC promoter (Wilson et al., 2013).

Recombinant lentiviruses were generated in 293 T cells transfected with the second-generation packaging vectors psPAX2 (Addgene#12260) and pMD2. G (Addgene#12259) using Lipofectin 2000 reagent (Invitrogen). Stably E2F1-Luc transfectant cells were selected with 400 μg/ml G418; stably NFkB-Luc transfectant cells were selected by FACS Sorting.

To assess the expression of miR200a,429,200b family cluster, cells were transiently transfected with pGL3–1574/+ 120, which encodes *Firefly Luciferase* downstream a segment of the human gene, encompassing − 1574 to + 120 relative to the putative transcription start site (TSS) (Bracken et al., 2008) of miR 200a, 200b, 429 family cluster. pGL3–1574/+ 120 was a gift from Greg Goodall (Addgene plasmid #

35539; http://n2t.net/addgene:35539; RRID:Addgene_35539). Transfection was performed using TransfeX Transfection Reagent (ATCC# ACS-4005™). Luciferase activity was measured on a IVIS 200 optical imager (Xenogen) either after selection for stable transfectants (lentiviral infection) or 48 h after transfection for transient transfectants.

### 2.8. Adipogenic and Osteogenic differentiation assays

Adipocyte differentiation was achieved with 2 weeks' culture of mesenchymal stem cells in adipogenic medium, containing 10–6 M dexamethasone, 10 μg/ml insulin, and 100 μg/ml 3-isobutyl-1-methylxantine (all from Sigma Immunochemicals, Milan, Italy). Osteoblast differentiation was achieved with 2 weeks' culture in osteogenic medium containing 10–7 M dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate (Sigma Immunochemicals). Oil Red O, and von Kossa, were used to identify adipocytes and osteoblasts, respectively.

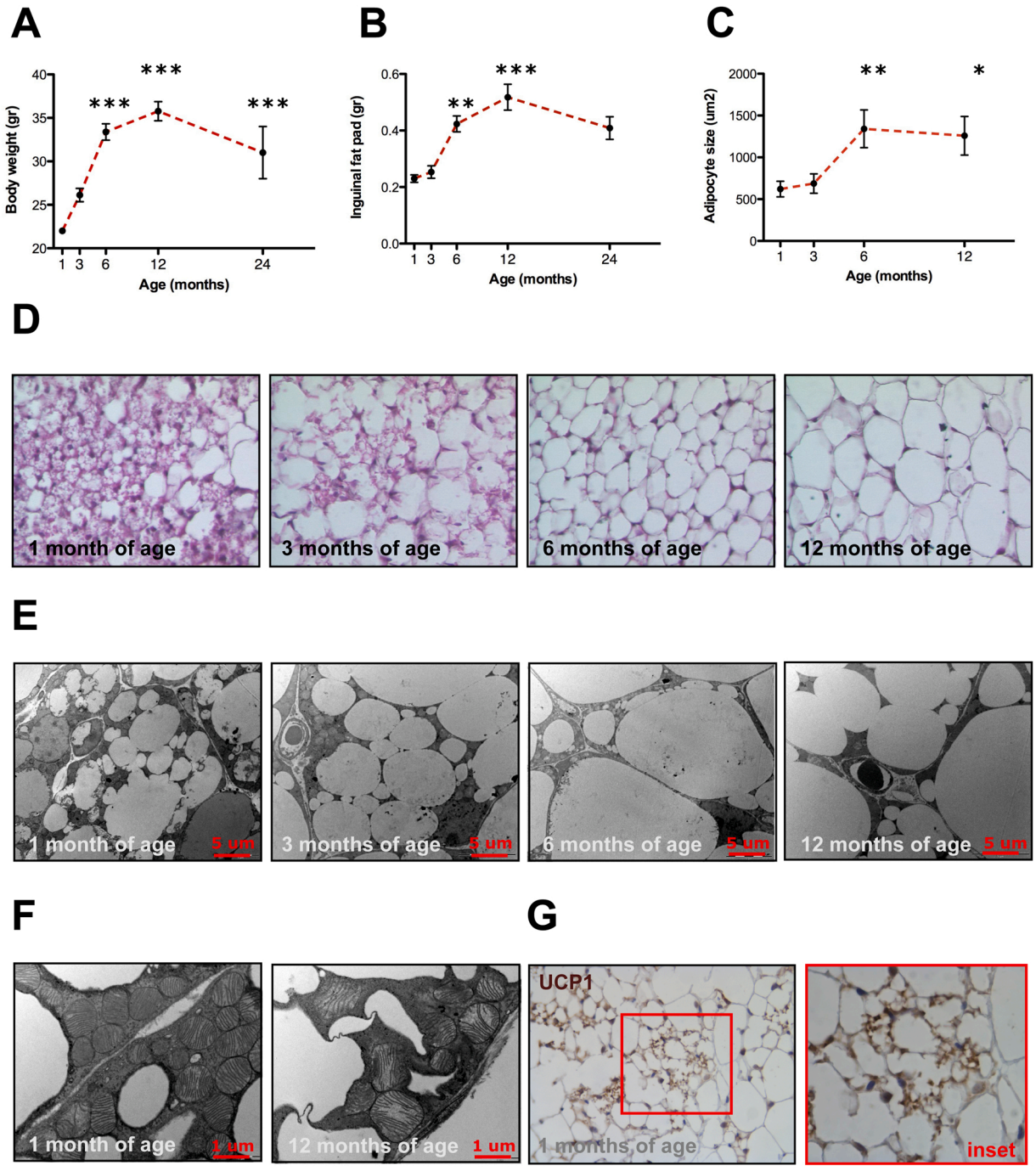


**Fig. 1.** Inguinal white adipose tissue (WAT) undergoes major anatomical changes with age. The body weight (A) and the weight of the inguinal fat pad (B) of mice progressively increased with age until middle age (12 months old), then slightly decreased. Adipocyte size also increased with age (C). The inguinal fat pad of young mice (1 month old) was enriched with brown-like multilocular adipocytes which were progressively replaced by white-like unilocular adipocytes with age as shown by optical (D) and electron microscopy (E). Mitochondria in the inguinal WAT of young mice were spherical and packed with laminar cristae, while this structure resulted degenerated in mitochondria of WAT from middle-aged mice (F). Clusters of multilocular adipocytes in young WAT proven to be positive for UCP1 (G).

## 3. Results

### 3.1. Brite adipocytes within inguinal white adipose tissue diminish with age

First, we confirmed the previously reported changes in the anatomical architecture of subcutaneous WAT with aging (Rogers et al., 2012). Inguinal fat pads from mice aged 1 (n = 8), 3 (n = 8), 6 (n = 8), 12 (n = 8) or 24 (n = 2) months were surgically extracted. Body weight (BW) and mass of the inguinal fat pads were found to progressively increase over adulthood until 12 months of age (Fig. 1A and B), while a slight decline was observed at 24 months of age. Expansion of WAT during adulthood was consistent with a remarkable increase in adipocyte size with age (Fig. 1C). Multilocular brown-like cells (Fig. 1D) with abundant cryst-rich mitochondria (Fig. 1E) were readily detectable in the inguinal WAT of young mice. Multilocular cells were progressively lost over adulthood and replaced by unilocular adipocytes, which displayed the typical morphology of "classic" white adipocytes (Fig. 1D and E). Multilocular cells likely represent brite adipocytes, which have been previously reported to be interspersed within subcutaneous WAT (Harms and Seale, 2013; Seale et al., 2008; Wu et al., 2012). They displayed the typical morphology of brown adipocyte, such as positivity for uncoupling proein 1 (UCP1) and mitochondria with spherical morphology and packed with laminar cristae (Fig. 1F and G).

The total number of SVF cells isolated *ex vivo* from the entire inguinal fat pad of mice of different ages remained unchanged (Fig. 2A), although SVF number per milligram of fat was progressively reduced likely due to the adipocyte enlargement (Fig. 2B). The abundance of clonogenic cells (colony-forming unit fibroblasts, CFU-F) per 10,000 SVF cells, did not significantly change with age (Fig. 2C). Taken together, these data suggest that subcutaneous WAT undergoes major changes in anatomical structure during adulthood, reaching a maximum weight and minimum fractional abundance of SVF/CFU-F cells at 12 months of age.

### 3.2. ASC from young mice display a "brown-like" signature which turns "white" with age

We wanted to assess whether the SVF cell phenotype might mirror

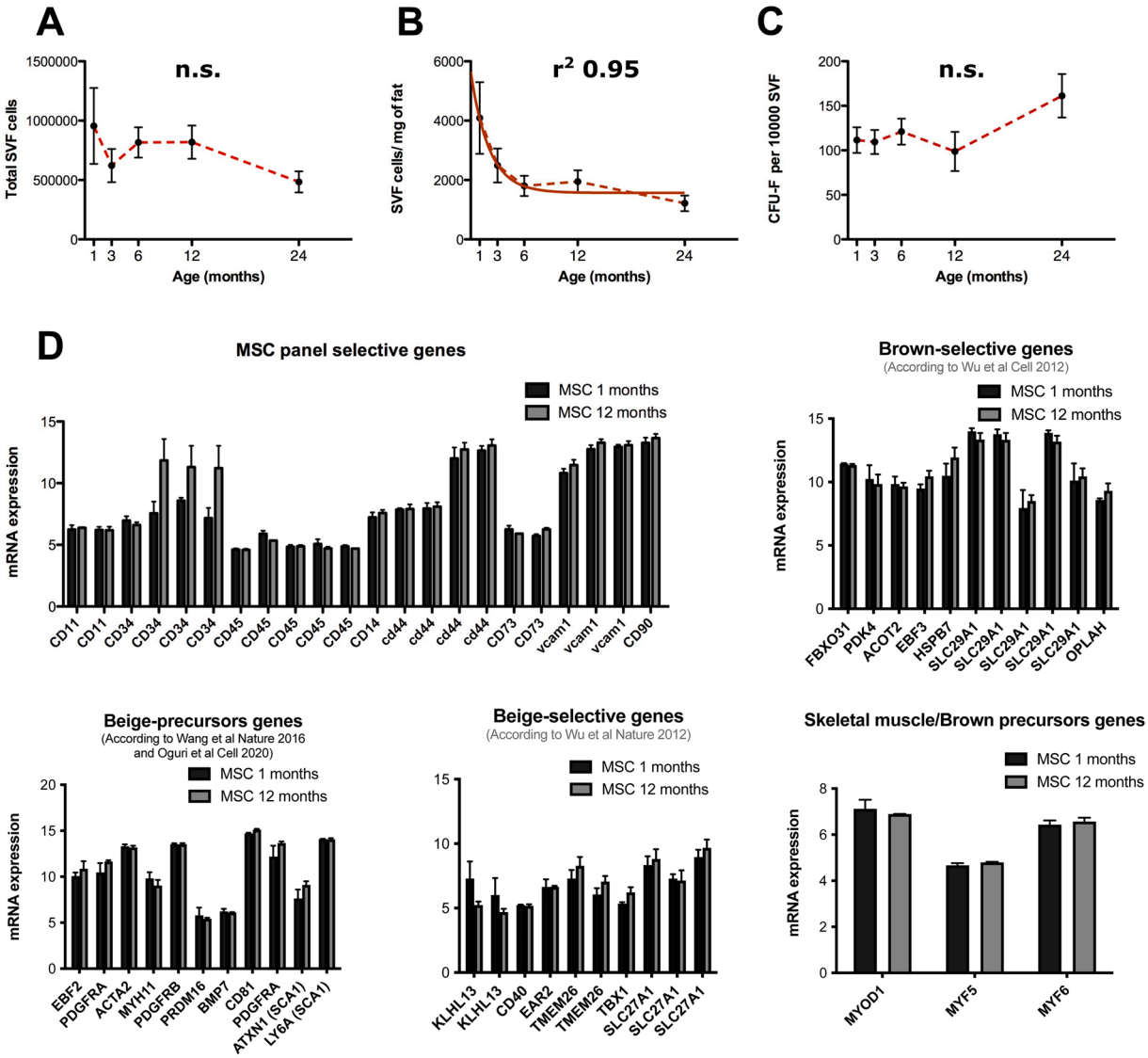


**Fig. 2.** Adipose-derived stromal cells (ASC) from inguinal WAT does not change with age either in number or in the expression profile of brown/beige precursor markers. The total count of stromal vascular fraction (SVF) cells in these WAT depots did not significantly changed with age (A). The number of SVF cells normalised to the weight of the respective depot exhibited a significantly decreasing trendline (B) (*** $p < 0.005$, ** $p < 0.01$). CFU-F fraction in SVF did not change with age (C). Adipose-derived stromal cells (ASCs) from inguinal WAT of 1- and 12-month-old mice did not exhibit any difference in the expression of either mesenchymal stem cell (MSC), brown or beige adipose tissue-specific markers (replicates on x-axis represents different microarray probes for the same gene) (D).

the anatomical changes of subcutaneous WAT with age. With this aim, we executed a whole-genome microarray analysis of ASC obtained by culturing SVF cells from 1- and 12-month-old mice. The ASC derived from younger (1 month) and older (12 months) mice exhibited a comparable pattern of transcriptional expression of mesenchymal stromal cell-related genes. No detectable difference was observed regarding the transcriptional expression of genes known to be specific to either differentiated or undifferentiated brown and beige lineages (Fig. 2D).

1mo and 12mo ASC did not show appreciable difference in proliferation rate (Fig. 3A) or senscence (Fig. 3B). 12mo ASC displayed a higher ability to adipogenic differention, while osteogenic differentiation ability was not different (Fig. 3C and D).

Timmons et al. (2007) previously revealed a remarkable divergence in the transcriptional profile between primary cultures of committed adipocyte precursors taken from either "classic" BAT or WAT depots. Hence, we were interested in investigating whether comparable differences might be recapitulated in ASCs as fat "whitens" with age. To achieve this, we compared our microarray data with the dataset of Timmons et al. (2007), retrieved from the Gene Expression Omnibus (GEO) database (GSE7032). The Timmons dataset comprises the transcriptional profile of committed pre-adipocytes from "classic" BAT and WAT depots, either undifferentiated or at the early stages of terminal differentiation. To define BAT and WAT precursor-specific signatures, we interrogated the Timmons dataset for transcripts with significantly ($p > 0.05$) different expression between undifferentiated BAT and WAT samples. We identified 1667 transcripts that were up-regulated in WAT precursors (WAT precursor signature) and 1194 up-regulated in BAT precursors (BAT precursor signature) (Fig. 4A).

Afterwards, we assessed the enrichment of these BAT and WAT precursor signatures on our microarray dataset of differently aged mice. This was achieved using gene set enrichment analysis (GSEA), which tests the statistical significance of small but coordinated changes in the expression of a priori defined gene sets (Mootha et al., 2003; Subramanian et al., 2005). The BAT precursor signature was significantly

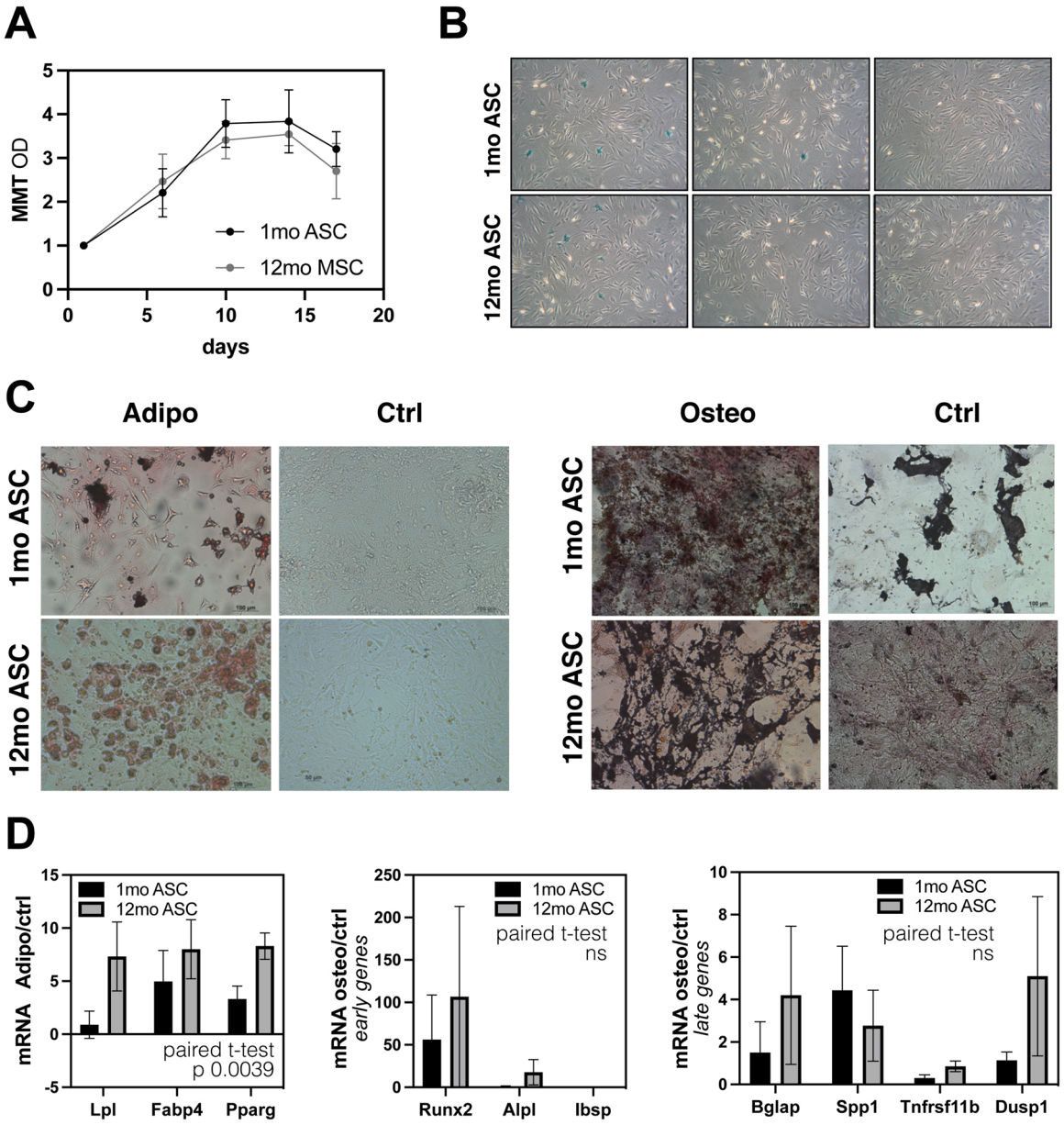


**Fig. 3.** Adipose-derived stromal cells (ASC) from middle-aged mice display an increased ability towards adipogenic differentiation. ASC established from inguinal WAT of mice of 1 or 12 months of age did not exhibit appreciable differences either in proliferation rate (A) or in senescence associated β-galactosidase staining (B). Young ASC exhibited a higher ability to adipogenic but not osteogenic differentiation, compared to middle-aged ASC (C and D).

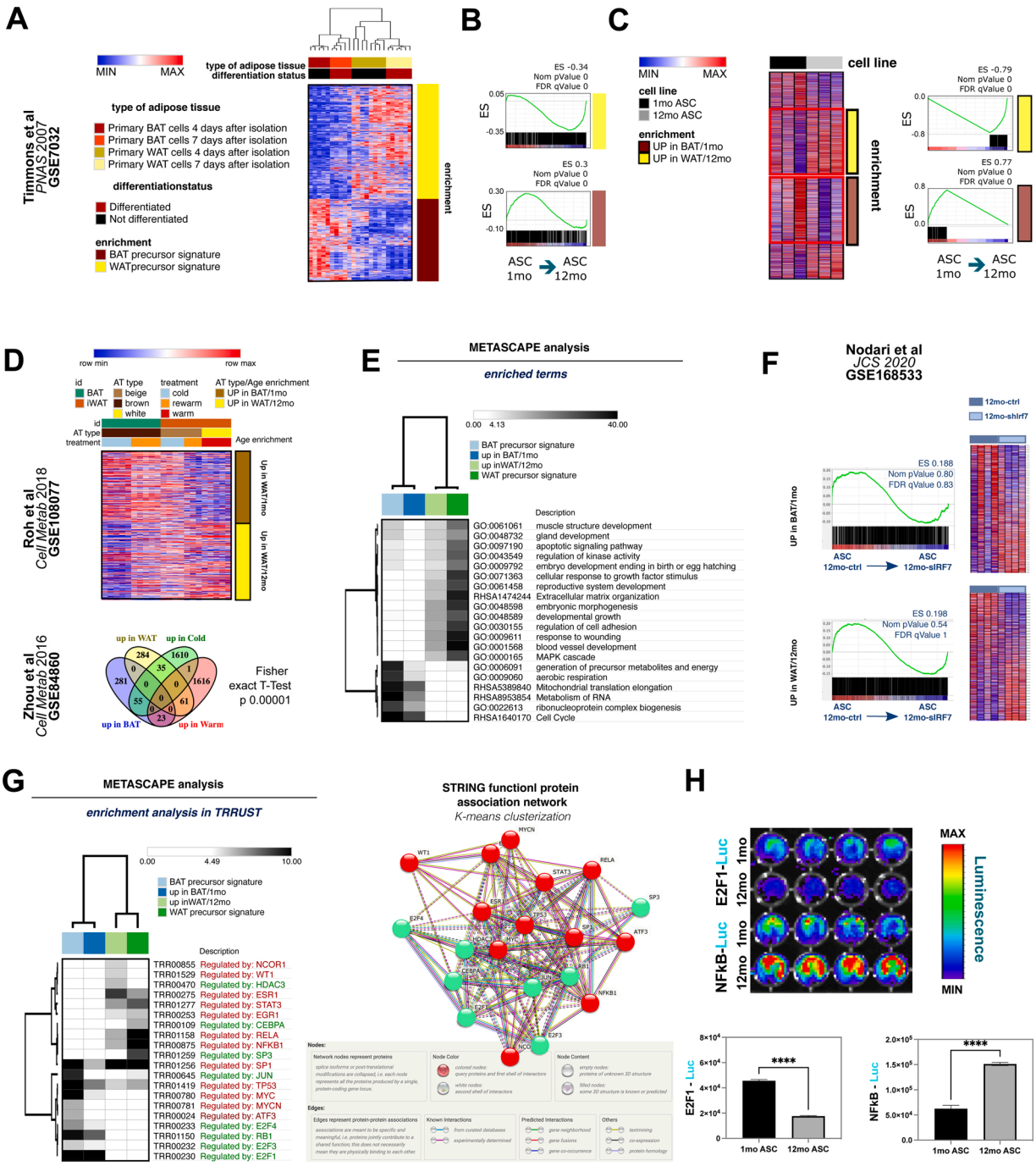


**Fig. 4.** Adipose-derived stromal cells (ASC) resident in white adipose tissue (WAT) switch from a brown- to white-like precursor transcriptional signature with age. Differential expression analyses of brown adipose tissue (BAT)- and WAT-derived precursor cells in the GEO-GSE7032 dataset identified multiple genes that were significantly (t-test $p < 0.05$) overexpressed or under-expressed in undifferentiated/early differentiated BAT precursor cells (BAT precursor signature) compared to undifferentiated/early differentiated WAT precursor cells (WAT precursor signature) (A). Gene set enrichment analysis (GSEA) revealed that the BAT precursor signature was enriched in ASCs from the inguinal WAT of 1-month-old mice, while the WAT precursor signature was enriched in ASCs from the inguinal WAT of 12-month-old mice (B). The precursor signatures of WAT and BAT were refined to include only the genes that displayed significant individual core enrichment in WAT-resident ASCs from either 1-month-old mice (UP in BAT/1mo signature) or 12-month-old mice (UP in WAT/12mo signature) according the GSEA metrics (C). UP in BAT/1mo and UP in WAT/12mo signatures were compared with adipose tissue cold-induced transcriptional changes reported by the studies of Roh et al. (GSE108077) and Zhu et al. (GSE84860) (D). Analysis with METASCAPE algorithm (Zhou et al., 2019) (https://metascape.org/gp/index.html#/main/step1) revealed that the UP in BAT/1mo signatures were mainly enriched in genes related to mitochondrial biogenesis and function (E). Knock-down of *Interferon Regulatory Factor 7 (Irf7)* did not change the expression profile of UP in BAT/1mo and UP in WAT/12mo signatures in 12mo ASC (F). TRRUST(Han et al., 2018) analysis through METASCAPE revealed two clusters of transcription factors the targets of which were differently enriched in UP in WAT/12mo or BAT/1mo signatures. Analysis with the STRING(Szklarczyk et al., 2017) database (https://string-db.org/) identified multiple interactions previously reported between these transcription factors (G). Luciferase assay of cells engineered with *Luciferase* gene under the control of either E2F1 or NFkB consensus binding sequence revealed a lower transcriptional activation by E2F1 and a higher transcriptional activity of NFkB in 12mo ASC compared to 1mo ASC (H).

enriched in ASC from younger (1 month of age) mice, while the WAT precursor signature was enriched in older (12 months of age) mice (Fig. 4B). Although overall enrichment of the gene sets proved to be statistically significant, not every gene of the two sets, taken individually, reached statistical power. Therefore, we refined the two gene sets to include only genes that exhibited significant individual core enrichment. We identified 380 genes that were upregulated in ASCs resident in the WAT of 12-month-old mice compared to those in the WAT of 1-month--old mice (UP in WAT/12mo), and 359 genes that were upregulated in ASC resident in the WAT of 1-month-old mice compared to those in the WAT of 1-month-old mice (UP in BAT/1mo) (Fig. 4C). To get rid the possibility that these signatures might reflect differences in fat depots of origin rather than intrinsic features of white/brown phenotypes, we compared our signature with 2 recently published datasets which provide the transcriptional change of WAT and BAT following cold-exposure (Roh et al., 2018; Zhu et al., 2016). Roh et al. (2018) profile the transcriptional portraits of forskolin-stimulated SVF cells explanted from either BAT or WAT depots of cold-induced (iWAT) and re-warmed mice. From the entire dataset we extracted the genes related to our UP in BAT/1mo and UP in WAT/12mo signatures. UP in BAT/1mo genes were clearly enriched in beige vs white phenotype, brown vs beige and cold vs re-warmed. In sharp contrast UP in WAT/12mo genes displayed a clearly inverse trend. Zhu et al. (2016) provide the list of genes up- or down-regulated in subcutaneous fat upon cold stimulation. Although our signatures only partially overlapped with these lists of genes, UP in WAT/12mo was significantly enriched in the list of genes upregulated in warm (61 vs 35), while UP in BAT/1mo was enriched in the list of genes upregulated in cold (55 vs 23). These data provide evidence that the transcriptional signature of tissue-resident ASCs consistently reflect the brown-to-white switch incurred by subcutaneous WAT with age.

### 3.3. WAT/12mo and BAT/1mo are enriched in targets of NfKB and E2F1 respectively

To go in depth into the molecular mechanisms underlying the transcriptional differences between BAT/1mo and WAT/12mo we assessed our signatures for the enrichment in functional categories according to the METASCAPE algorithm (Zhou et al., 2019). Genes "UP in BAT/1mo" were enriched in functional categories related to mitochondrial components and function (Fig. 4E). In a recent paper we identified the transcription factor *Interferon regulatory factor 7 (Irf7)* as master regulator of mitochondrial biogenesis and function (Nodari et al., 2021). To test the role of this transcription factor in the regulation of the brown/white-associated profiles we re-analysed our microarray dataset of *Irf7* knock-down 12mo ASC. Strikingly, we found that the down-regulation of *Irf7* failed to revert the expression profile of either WAT or BAT (Fig. 4F), indicating that other transcription factors are likely implicated in regulating these "UP in WAT" or "UP in BAT"-specific genes. Hence, we interrogated METASCAPE to identify transcription factors potentially interacting with the genes included in our signatures. Analysis through the TRRUST algorithm (Han et al., 2018) revealed two clusters of highly interacting transcription factors the targets of which were differently enriched in "UP in WAT/12mo" or "UP in BAT/1mo" signatures. Specifically, "UP in WAT/12mo" signatures was mainly enriched in targets of NFkB-RELA, while "UP in BAT/1mo" signatures were mainly enriched in targets of E2F1-RB1 complexes of transcription factors (Fig. 4G). In line with our data, the E2F1-RB1 complex of transcription factors has been previously shown to suppress oxidative metabolism in brown adipose tissue (Blanchet et al., 2011). To comparatively assess the functional activity of E2F1 and NFkB transcription factors in 1mo vs 12mo ASC we engineered these cells with the *Luciferase* reporter genes under the consensus binding sequence of these transcription factors. As expected, 1mo ASC displayed higher E2F1-driven and reduced NFkB-driven Luciferase activity compared to 12mo ASC (Fig. 4H).

### 3.4. MicroRNAs related to "beiging" in adipose-derived stromal cells diminish with age

Extensive rearrangement of microRNA processing has been suggested to underlie both the aging (Mori et al., 2012) and whitening (Mori et al., 2014) of adipose tissue. Specifically, massive down-regulation (Mori et al., 2012) of multiple microRNAs has been recently implicated in the whitening of brown adipocyte precursor cells immortalised in vitro (Mori et al., 2014). To assess the potential role of miRNAs in the age-related transcriptional changes of WAT-resident ASCs, we profiled the expression of 502 mouse-specific miRNAs (as listed in miRBase release 9.2) on ASCs isolated from the inguinal WAT of mice aged 1, 3, 6, 12 or 24 months. The fraction of miRNAs inversely ($r^2 < -0.7$, $p < 0.05$) correlated with age significantly exceeded that of miRNAs that were positively ($r^2 > 0.7$, $p < 0.05$) correlated with age (11% vs 5%, Fisher t-test, $p = 0.00036$) (Fig. 5A). Most miRNAs whose targets have been previously implicated in the whitening of brown preadipocytes (Mori et al., 2012, 2014) displayed a strong negative correlation with age (Fig. 5A), and their validated targets (as identified in miRTarBase, http://mirtarbase.mbc.nctu.edu.tw; "Targets miRNA DOWN") were consistently increased in ASCs derived from 12-month--old mice compared to 1-month-old mice (GSEA ES 0.3 *FDR 0.0*). The list of these "Targets miRNA DOWN" incudes a significantly higher fraction of genes belonging to UP in WAT/12mo than UP in BAT/1mo (9% vs. 2.2%, Fisher t-test $p = 3.1 \times 10^{-5}$) (Fig. 5B). To validate the mirRNA profiling data we engineered 1mo and 12mo ASC with the *Luciferase* gene under control of the promoter region of *mir200A, 429, 200B* family cluster. According to microarray data, luciferase activity resulted significantly higher in 1mo vs 12mo MSC. Afterwards, we interrogated Transmir 2.0 algorithm (Tong et al., 2019) to identify potential transcription factors (TFs) underlying the repatterning of miRNA profile with age. Strikingly, miRNA positively correlated with age (Correlation Index, $Ci>0.7$, $p < 0.05$) did not show significant ($FDR<0.05$) association with any TF. In sharp contrast, miRNA negatively correlated with age ($Ci<-0.7$, $p <= 0.05$) displayed association with many TFs, including NFkB ($p = 0.00$), RELA ($p = 0.03$) and, to a lesser extent, E2F1 ($p = 0.09$).

Collectively, these data are consistent with the notion that the age-related whitening of WAT-resident ASCs relies, at least in part, on extensive downregulation of multiple miRNAs and that the miRNA repatterning with age might involve the same TFs potentially involved in age-related mRNA transcriptional changes.

### 3.5. Age-related white adipose tissue signature relates metabolic dysfunction

The agents that induces the "browning" of WAT is associated with metabolic benefits.[3,] (Liu et al., 2015) On the other hand, depletion of the function of beige adipocytes in subcutaneous fat leads to metabolic dysfunction, such as obesity and insulin resistance (Cohen et al., 2014). Hence, we wanted to assess whether the UP in BAT/1mo and UP in WAT/12mo signatures could identify pathophysiological states in human patients by taking advantage of publicly available databases.

According to the HuGE Navigator and EMBL Expression Atlas open-source databases, approximately double the genes in the UP in WAT/12mo signature compared to UP in BAT/1mo signature overlapped with the lists of genes associated with diabetes and obesity (Fig. 6A), although the fraction of overlapping genes was low for both signatures. Afterwards, we assessed the overlap of our signatures with those of two studies, the first of which provided the transcriptional profiles of adipose tissue and skeletal muscle stratified for insulin sensitivity and responsiveness to thiazolidinedione (TZD)-based treatment, which is an insulin-sensitizing drug used to treat type 2 diabetes Sears et al. (2009), (Gene Expression Ominbus database, accession GSE13070) (Fig. 6B), and the second provided the profiles of adipose tissue and blood of normal and obese patients Drong et al. (2013), (ArrayExpress Database,

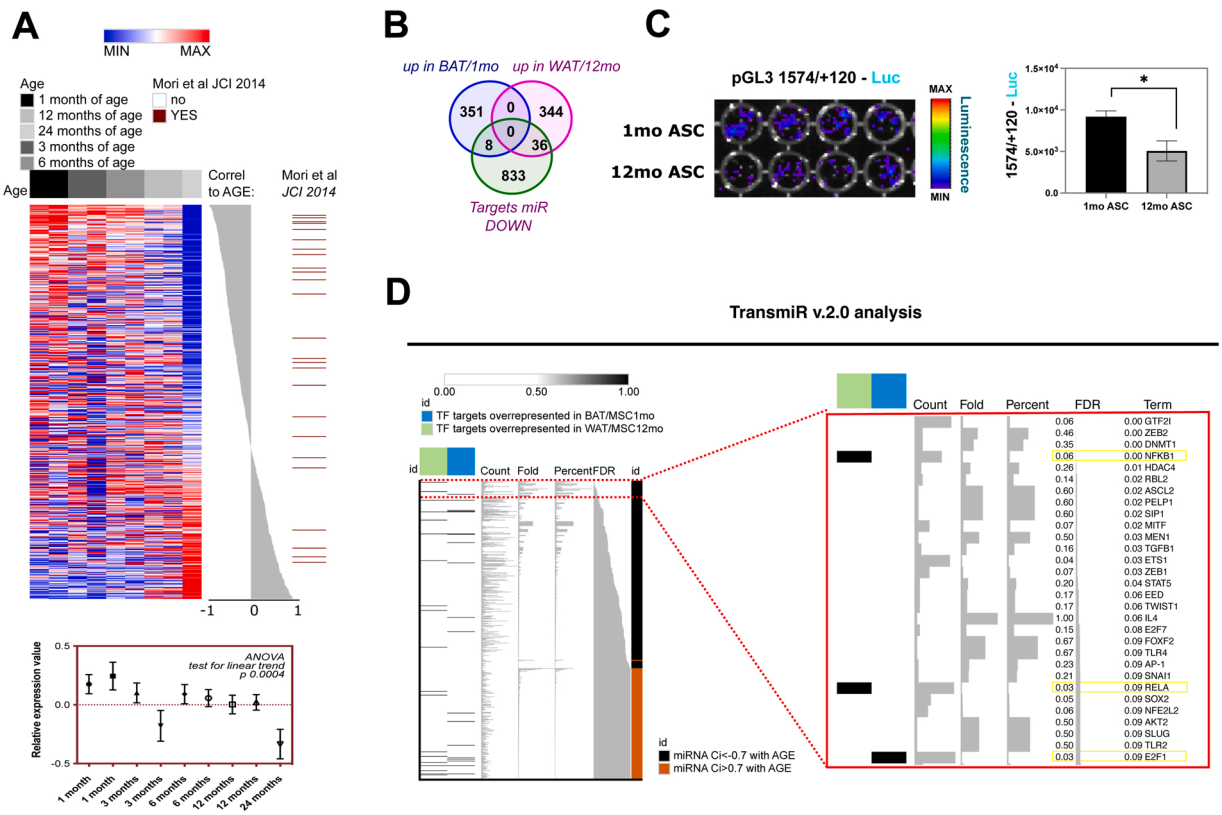


**Fig. 5.** Aging of the adipose-derived stromal cells (ASC) resident in white adipose tissue (WAT) brings about an overall down-regulation of miRNAs. The microarray profile of miRNA expression in ASCs taken from the inguinal WAT of mice aged 1, 3, 6, 12 or 24 months showed an overall decline of miRNA with age. The list of miRNAs that were downregulated with age included many of the miRNAs the depletion of which was previously shown to be implicated in the brown-to-white switch of adipocyte precursors (Mori et al., 2014) (A). Previously validated targets of these miRNAs according to miRtarBase (Targets miRNA down) were overrepresented in the transcriptome of WAT-resident ASCs from 12-month-old mice compared to 1-month-old mice (B). Luciferase assay of ASC engineered with *Firefly luciferase* gene downstream a segment of the human gene, encompassing − 1574 to + 120 relative to the putative transcription start site (TSS) (Bracken et al., 2008) of miR200a, 200b,429 family cluster revealed a higher transcriptional activity of this cluster in 1mo ASC compared to 12mo ASC (C). *In silico* anaysis of transcription factor binding site enrichment through TransmiR 2.0 algorithm (Tong et al., 2019) (http://www.cuilab.cn/transmir) revealed that miRNA significantly anticorrated with age (Correlation index, Ci<−0.7, $p < 0.05$) were targets of a limited number of transcription factors, including NFkB, RELA and E2F1 (D).

accession E-MTAB-54) (Fig. 6C). For both study datasets, we compiled a matrix displaying the enrichment scores (calculated using the GSEA algorithm (Subramanian et al., 2005)) of the "UP in BAT/1mo" or "UP in WAT/12mo" signatures between each sample pair (blue-red heatmap) and their corresponding p-value (yellow-black heatmap). The "UP in WAT/12mo" genes were strongly enriched in adipose tissue compared to skeletal muscle, insulin-resistant adipose tissue (red arrowhead) compared to non-insulin-resistant tissue (blue arrowhead) (Fig. 6B), in adipose tissue compared to blood, and in the adipose tissue (both abdominal and gluteal) of obese males compared to non-obese males (Fig. 6C). The "UP in BAT/1mo" genes displayed a much smaller difference between samples with low/moderate upregulation in skeletal muscle compared to adipose tissue, in insulin-resistant adipose tissue responsive to TZD compared to non-responsive tissue (Fig. 6B), in adipose tissue compared to blood, and in gluteal fat compared to abdominal fat (Fig. 6C). These results indicate that the transcriptional signature of ASCs from the WAT of middle-aged mice reflects a phenotype of metabolic dysfunction, which includes insulin resistance and obesity.

Collectively, our data demonstrate that ASCs within subcutaneous WAT depots undergo major transcriptional changes with aging, which consistently reflect the “whitening” of the surrounding WAT along with the loss of embryonic genes related to cell cycle and DNA repair (Fig. 7).

## 4. Discussion

Adipose tissue has long been considered an undiversified fat-storage that is uniformly distributed throughout the body. Two decades of studies have unveiled the complex biology behind the different “shades” of adipose tissue (Peirce et al., 2014) and their major role in coordinating metabolic homeostasis (Scherer, 2006). However, much remains to be clarified, especially regarding the stem cell nature and the role of the precursor cells which populate the SVF of adipose tissue in adults. (Hepler et al., 2017) Due to their regenerative and immunomodulatory properties, these cells have garnered enormous interest as a potential therapeutic tool in a variety of clinical settings, including plastic and reconstructive surgery, cartilage and bone regeneration, treatment of cardiac dysfunction and autoimmune diseases, among others (Constantin et al., 2009; Grayson et al., 2015; Mizuno et al., 2012; Roura et al., 2017; Wu et al., 2013). A large body of studies have focused on whether the donor age affects the specific therapeutic potential of ASCs. Nevertheless, the inherent biology of ASCs, their role in the context of adipose tissue function and how their phenotype reflects the pathophysiological changes of the fat depot they reside in, remain scarcely understood (Hepler and Gupta, 2017; Hepler et al., 2017). Herein, we show that ASCs resident in classic WAT undergoes global transcriptional re-patterning with age which mirrors the progressive “whitening” of their fat origin. Previous reports have shown that brite adipocyte clusters resident within subcutaneous WAT progressively diminish during adulthood (Rogers et al., 2012). Our data complement this notion, demonstrating that the transcriptional phenotype of ASCs reflect this age-related brown-to-white switch.

The origin of beige adipocytes in adults is currently subject of intense

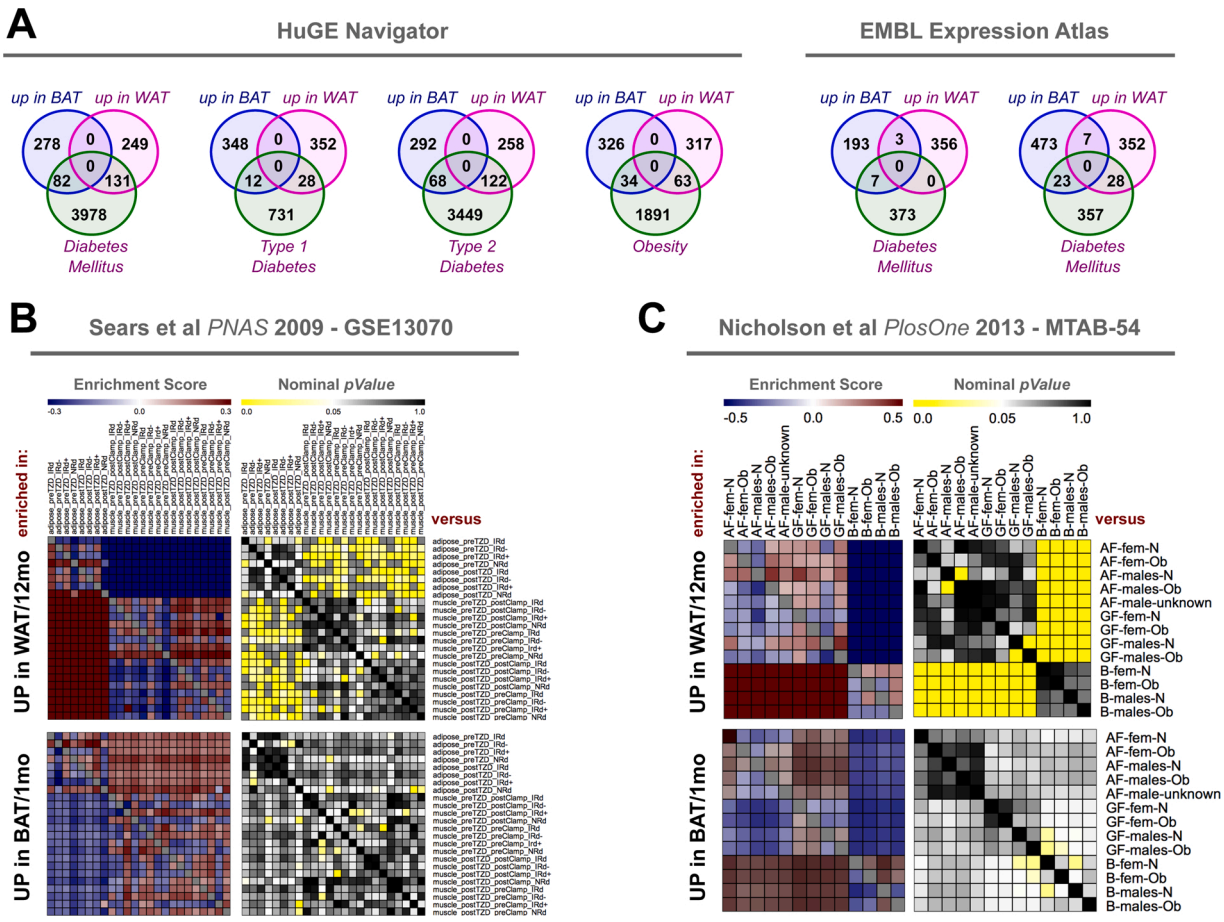


**Fig. 6.** Transcriptional "whitening" of white adipose tissue (WAT)-derived adipose-derived stromal cells (ASC) is correlated with the transcriptional signatures associated with metabolic disorders. The UP in WAT/12mo signature included around double the number of genes associated with metabolic disease, such as type 1 and 2 diabetes mellitus (DM), compared to the UP in BAT/1mo signature, as identified in the HuGE Navigator and EMBL Expression Atlas databases (A). The enrichment of UP in BAT/1mo and UP in WAT/12mo signatures were evaluated between pairs of samples from the Sears et al. (B) and Nicholson et al. (C) datasets, which include microarray profiles of human subjects with the indicated features and clinical conditions. Blue-red heatmaps display the enrichment scores (ES) between the indicated pairs of samples, while the yellow-red heatmaps display the respective p-values according to the gene set enrichment analysis (GSEA) algorithm.

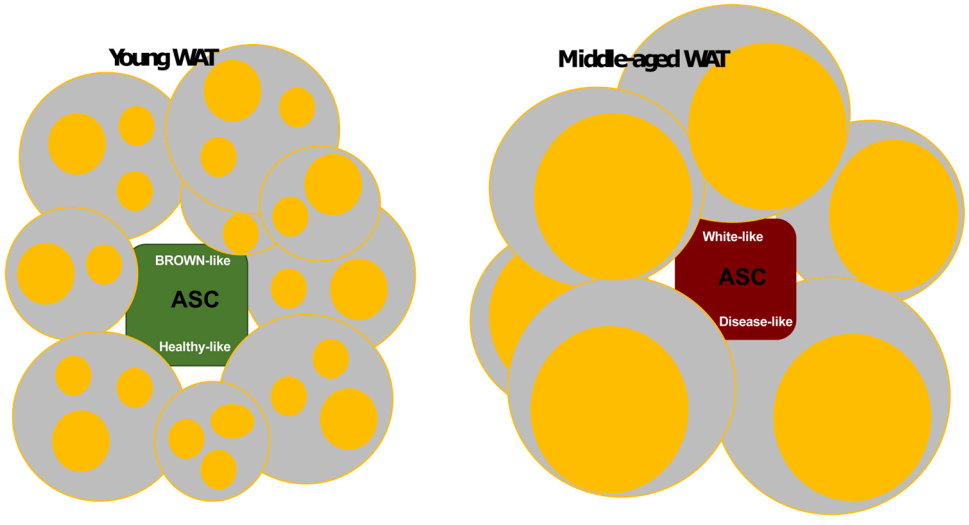


**Fig. 7.** Adipose-derived stromal cell (ASC) transcriptome reflects physiological and pathological changes of WAT with aging. The WAT from young mice contains ASC exhibiting a brown-like and healthy-like transcriptional signature which disappear with aging.

debate (Hepler and Gupta, 2017; Ikeda et al., 2018; Wang and Seale, 2016). The most accredited model is that beige adipocyte may be induced by environmental inputs (cold, sympathetic stimulation, exercise, and others) either through *de novo* differentiation from fat-resident dormant-precursors or from trans-differentiation from mature white adipocytes. Noteworthy, beige precursors are thought to have a different developmental origin with respect to brown adipocyte and have the unique ability to transition between brown-like to white phenotype. Several studies in the last years have contributed to identify specific markers capable to discriminate brown/beige and white specific precursors (Oguri et al., 2020; Seale et al., 2008; Wu et al., 2012). ASC from differently aged mice did not show detectable difference in the expression patterns of these set of genes. This might reflect the fact that these markers snapshot the identity of beige, brown and white precursors in discrete states of commitments toward their respective differentiation fate, while ASC represent an heterogenous population of cells at an earlier, still uncommitted state. Beyond thermogenic function, brown/beige phenotype is gaining much attraction for its established association with healthy metabolic homeostasis (Cohen et al., 2014; Harms and Seale, 2013). Our data suggest that the identity of brown/-beige phenotypes are intrinsically retained in still undifferentiated ASC population in youth, but it is progressively lost with age. The emerging progress of adipose tissue stromal cells at single-cell resolution will help to dissect the heterogeneity of subcutaneous fat precursors and the differentiation programs they may execute following changes of environmental conditions (Wang et al., 2021).

Functional analysis of the differences in mRNA and microRNA profiles between BAT/1mo and WAT/12mo revealed a clear-cut distinction in the enrichment of candidate regulatory elements. This suggests the existence of the transitional states which might switch one-to-another upon the activation of antagonistic transcription factor programs. These include E2F1-RB1 complexes and NFkB-RELA. E2F1-RB1 complex has been previously shown to play a central role in suppressing oxidative metabolism in brown adipose tissue and its loss-of-function preserves mice from diet induced obesity (Blanchet et al., 2011). In line with this notion, we found that the transcriptional activity of E2F1-specific promoter is readily reduced with aging in ASC, which may contribute to the whitening of inguinal fat with age. NFkB has long been implicated in low-grade inflammation which occurs with aging at both systemic (inflammaging) (Franceschi et al., 2018) and in adipose tissue (adipaging) level (Pérez et al., 2016). However, its role in regulating the transition between the different "shades" of fat is still not clarified. Our data suggest a pivotal role for NFkB transcription factor in regulating the transcriptional network (both mRNA and microRNA) underneath the progressive "whitening" of adipocyte precursors over the adult life.

## CRediT authorship contribution statement

**Ilaria Scambi:** Investigation. **Daniele Peroni:** Investigation, Writing – review & editing. **Alice Nodari:** Investigation, Writing – review & editing. **Flavia Merigo:** Investigation. **Donatella Benati:** Investigation. **Federico Boschi:** Investigation. **Silvia Mannucci:** Investigation. **Andrea Frontini:** Investigation, Writing – review & editing, Data curation. **Silvia Visonà:** Investigation, Writing – review & editing. **Andrea Sbarbati:** Writing – review & editing, Funding acquisition, Data curation. **Mauro Krampera:** Investigation, Writing – review & editing, Funding acquisition, Data curation. **Mirco Galiè:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition, Data curation, Supervision.

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*Additional informations*

Authors have no competing financial interests in relation to the work described.

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