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Editor's Summary

Fat Cells Gain New Identities

There's "good fat" and there's "bad fat." Good fat is considered to be brown adipose tissue (BAT), which burns calories. Bad fat can be white adipose tissue (WAT), which stores lipids as energy and, in excess, contributes to obesity. When brown fat cells, or adipocytes, develop within white fat, they are called "beige." Sorting out these different adipocyte subtypes within the human body has been challenging but will be important in uncovering the underlying mechanisms for obesity and its comorbidities, such as type 2 diabetes. To this end, Ussar and colleagues have now identified three new surface markers of white, beige, and brown fat cells. These markers—ASC-1, PAT2, and P2RX5—were first selected in silico, then confirmed in mouse WAT and BAT, and lastly verified in human adipose tissue biopsies. ASC-1, PAT2, and P2RX5 are located in the plasma membrane of adipocytes, thus making them prime targets for imaging fat locations within the body and for directing therapeutics toward particular fat depots.

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CELL BIOLOGY

ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes

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White, beige, and brown adipocytes are developmentally and functionally distinct but often occur mixed together within individual depots. To target white, beige, and brown adipocytes for diagnostic or therapeutic purposes, a better understanding of the cell surface properties of these cell types is essential. Using a combination of in silico, in vitro, and in vivo methods, we have identified three new cell surface markers of adipose cell types. The amino acid transporter ASC-1 is a white adipocyte–specific cell surface protein, with little or no expression in brown adipocytes, whereas the amino acid transporter PAT2 and the purinergic receptor P2RX5 are cell surface markers expressed in classical brown and beige adipocytes in mice. These markers also selectively mark brown/beige and white adipocytes in human tissue. Thus, ASC-1, PAT2, and P2RX5 are membrane surface proteins that may serve as tools to identify and target white and brown/beige adipocytes for therapeutic purposes.

INTRODUCTION

Obesity and its comorbidities are a growing burden to our health and health care system. Fundamental to the development of obesity is an imbalance between caloric intake and energy expenditure, which results in accumulation of lipids within white adipose tissue (WAT) depots. This is, in part, countered by brown adipose tissue (BAT) that can burn excessive calories via uncoupled respiration using mitochondrial uncoupling protein-1 (UCP-1) (*1*, *2*).

In addition to classical BAT occurring in selected depots, brown adipocytes can develop within WAT and intramuscular fat upon chronic cold or β3-adrenergic receptor stimulation (*3*). These "beige" or "brite" fat cells originate from precursor populations distinct from classical brown fat (*4*–*8*) and exhibit different gene expression patterns from both brown and white adipocytes (*7*, *9*, *10*). These cells, as well as perhaps other unidentified subpopulations of white adipocytes, contribute to the heterogeneity of white fat depots and potentially to the different metabolic risk associated with accumulation of visceral versus subcutaneous fat (*11*).

Defining adipose depot composition with regard to white, brown, and beige adipocyte cell lineages is especially difficult in humans, where brown/beige fat is located in deeper cervical, supraclavicular, and paraspinal areas (*12*–*14*) and consists of a mixture of brown and white adipocytes. Recent studies suggest that in humans, these depots may contain a mixture of classical brown and beige adipocytes, depending on the exact location of the depot or depth within the cervical region (*7*, *15*, *16*).

As noted above, white, brown, and beige adipocytes differ greatly in their function. An increase in WAT, especially visceral fat, underlies the development of insulin resistance and the metabolic syndrome. In contrast, increasing the amount or activity of brown/beige fat may help improve caloric balance and metabolism and serve as a pathway for the treatment of obesity and its complications. However, because most adipose-related proteins are common to white and brown adipocytes, many of the currently used drugs intended to modulate WAT function can also affect BAT and conversely. Thus, novel strategies are needed to selectively target white and brown/beige adipose tissue.

A number of marker genes have been identified to distinguish brown, beige, and white adipocytes (*7*, *10*, *17*). These include *leptin*, *HOXC8*, and *HOXC9* for white fat; *TBX1* and *TMEM26* for beige fat; and *UCP-1*, *CIDEA*, and *PRDM16* for brown fat. These genes have been useful for the characterization of specific isolated adipocyte subtypes, but their usefulness in identifying and targeting distinct adipocyte types in intact tissue or in vivo is limited (*9*, *15*) because most of these markers represent intracellular or secreted proteins. Furthermore, some of these markers, such as *PRDM16*, are also expressed in non-adipose tissues (*18*). Thus, specific surface markers would be very helpful in identifying and targeting different adipocyte subpopulations in vivo.

Here, we have identified specific cell surface markers for white, brown, and beige adipocytes using a combination of in silico, in vitro, and in vivo approaches. These markers provide novel tools to identify different adipocyte populations in both humans and rodents and target them for therapy in vivo.

RESULTS

In silico identification of white adipocyte surface markers

Adiponectin is the most abundant and specific adipocyte protein (*18*). However, it is primarily a secreted protein (adipokine), making it of limited value for identification of intact adipocytes in vivo. To identify adipocyte-specific or enriched cell surface proteins, we used the expression pattern of mouse *adiponectin* as a model and searched the SymAtlas database (http://www.biogps.org) that contains data from multiple microarray experiments for genes whose expression correlated with *adiponectin* with a coefficient greater than 0.95 (fig. S1, A and B). This list was then filtered for genes with high expression in fat

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(more than three times the mean of all other tissues) that also had lower than mean levels of expression in skeletal muscle, heart, lung, pancreas, cerebellum, and cerebral cortex. This yielded three transmembrane proteins: *N*-acetyltransferase 8-like (*Nat8l*), prolactin receptor (*Prlr*), and neuregulin-4 (*Nrg4*). Of these three, *Nrg4* showed the highest adipose tissue specificity with a correlation coefficient to *adiponectin* of >0.95 (fig. S2 and http://www.biogps.org).

To increase the number of adipocyte-specific surface proteins, we repeated the search for genes whose expression also correlated with *Nrg4*. This identified six additional candidate genes as "adipocyte-specific" membrane proteins: transmembrane protein 120B (*Tmem120B*), adrenergic β3 receptor (*Adrb3*), aquaporin-7 (*Aqp7*), G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptor 81 (*Gpr81*), fatty acid transporter (*Slc27a1/Fatp1*), and solute carrier family 7 member 10 (*Slc7a10/Asc-1* neutral amino acid transporter, y+ system) (fig. S2 and http://www.biogps.org).

Asc-1 as white adipocyte surface marker in mice

To confirm the Affymetrix mouse protein expression data, we generated a complementary DNA tissue library containing 18 tissues from 7-week-old C57BL/6 male mice, as well as uterus and ovary from female mice, and used this to screen all candidate genes. Mice were perfused with phosphate-buffered saline (PBS) before sacrifice to minimize contamination of the tissues with blood cells. Although all tested genes showed a significantly higher expression in adipose tissue compared to other tissues, *Nrg4*, *Aqp7*, and *Asc-1* showed the greatest adipose tissue specificity (fig. S2).

Adipose tissue is composed of adipocytes and a stromal vascular fraction (SVF) including blood cells, immune cells, endothelial cells, and preadipocytes. To test for the adipocyte specificity of the selected candidate genes, we isolated RNA from purified mouse adipocytes and SVF from several fat depots. Whereas SVF had higher or similar expression of *Gpr81*, *Adrb3*, and *Aqp7* when compared to adipocytes from all adipose depots (fig. S3), *Nrg4*, *Fatp1*, *Tmem120B*, and *Asc-1* (fig. S3 and Fig. 1A) were specific to adipocytes. *Asc-1* expression was significantly higher in white adipocytes than in brown adipocytes or the SVFs (Fig. 1A). In addition, *Asc-1* showed about fivefold higher expression in adipocytes from WAT depots than from BAT (Fig. 1A). By comparison, there were no differences in *Nrg4*, *Fatp1*, and *Tmem120B* expression between white and brown adipocytes (fig. S3).

The unique expression pattern for *Asc-1* was confirmed in a second panel of 45 tissues isolated from 7-week-old C57BL/6 mice perfused with PBS, with the highest expression of *Asc-1* in perigonadal and perirenal, followed by subcutaneous and mesenteric WAT (Fig. 1B), all of which were equal to or higher than brain—the tissue previously reported to most highly express *Asc-1* (*19*, *20*). Low expression of *Asc-1* was observed in interscapular brown fat, adrenal gland, pancreas, and lung (Fig. 1B). The difference in *Asc-1* expression between white and brown fat ranged from 4-fold (mesenteric fat to interscapular brown fat) to 79-fold (perigonadal fat to subscapular brown fat) (Fig. 1B).

The expression of *Asc-1* was unaltered in WAT of the obese, leptin receptor–deficient *db*/*db* mice compared to lean *db*/+ controls (Fig. 1C), but increased in BAT of obese *db*/*db* mice, as well as in subcutaneous WAT of obese leptin-deficient *ob*/*ob* mice compared to *ob*/+ mice (Fig. 1D). Wild-type mice fed a high-fat diet (HFD) containing 60% calories of fat for 8 weeks also had significantly increased expression of *Asc-1* in BAT compared to chow diet (CD)–fed animals (Fig. 1E). The increases in *Asc-1* in BAT are consistent with the accumulation of white adipocytes in BAT in obese mice.

Fluorescence-activated cell sorting (FACS)–sorted primary murine preadipocytes from subcutaneous and perigonadal WAT (*21*) expressed very low levels of *Asc-1* compared to mature, in vitro–differentiated adipocytes derived from these cells (Fig. 1F). Likewise, we were unable to detect *Asc-1* expression in either cultured and differentiated primary brown preadipocytes or white adipocytes, further indicating that *Asc-1* is a marker of mature white adipocytes. This white adipocyte specificity was lost after immortalization, such that brown adipocytes derived from the SVF of BAT using SV40 large T antigen began to express this white adipocyte marker (fig. S4).

Western blots of tissue lysates from mouse subcutaneous and perigonadal WAT, as well as BAT, confirmed the expression of ASC-1 in white, but not brown, fat (Fig. 1G). Immunofluorescence staining for ASC-1 in differentiated, immortalized murine white adipocytes revealed cell surface staining (Fig. 1H), and this was confirmed by Western blotting after membrane fractionation of murine adipose tissues (Fig. 1I).

Like FACS-sorted primary murine white preadipocytes, cultured primary human subcutaneous preadipocytes had low levels of *ASC-1*, but expression was induced upon differentiation to mature adipocytes in vitro (Fig. 1J). *ASC-1* gene expression in human adipocytes was similar to the expression pattern of *leptin*, a classical white adipocyte marker (Fig. 1J).

Identification of brown adipocyte cell surface markers

To identify brown adipocyte–specific surface proteins, we performed a similar database search as shown in fig. S1A using the brown adipocyte–specific protein *Ucp-1* as a reference gene (fig. S1C). This identified two potential brown fat–specific transmembrane proteins: G protein–coupled receptor 119 (*Gpr119*) and proton assistant amino acid transporter-2 (*Pat2*). On further analysis, *Gpr119* was not unique to BAT and was similar in adipocytes and the SVF (figs. S5 and S6). By contrast, *Pat2* expression was mostly restricted to mouse adipose tissue, with 3.5- to 7.5-fold greater expression in BAT than in WAT depots, and very low levels in heart, lung, gallbladder, pancreas, and adrenal gland (Fig. 2A). Upon cell fractionation, *Pat2* expression was restricted to adipocytes, with minimal expression in the SVF, and was significantly more highly expressed in brown than in white adipocytes (Fig. 2B). Likewise, *Pat2* expression was very low in immortalized murine brown preadipocytes, but its expression was highly induced upon differentiation (Fig. 2C). *Pat2* expression was also observed in white adipocytes differentiated from immortalized subcutaneous mouse SVF (fig. S4), indicating again that these brown and white adipocyte markers were no longer specific in these immortalized cells.

As with *Asc-1*, there were no differences in the very low levels of *Pat2* expression in white fat of obese *db*/*db* mice compared with *db*/+ mice (Fig. 2D). However, there was a moderate down-regulation of *Pat2* in *db*/*db* mice compared to *db*/+ mice (Fig. 2D), suggesting a decrease in brown adipocytes at the expense of white adipocytes. Western blots of subcutaneous and perigonadal WAT and interscapular BAT from C57BL/6 mice showed detectable levels of PAT2 only in BAT (Fig. 2E). Immunostaining of PAT2 in brown adipocytes confirmed the surface localization of PAT2 (Fig. 2F), and this was further validated by membrane fractionation (Fig. 2G).

We performed another database query with *Pat2* as a reference gene and identified four additional transmembrane proteins on mouse

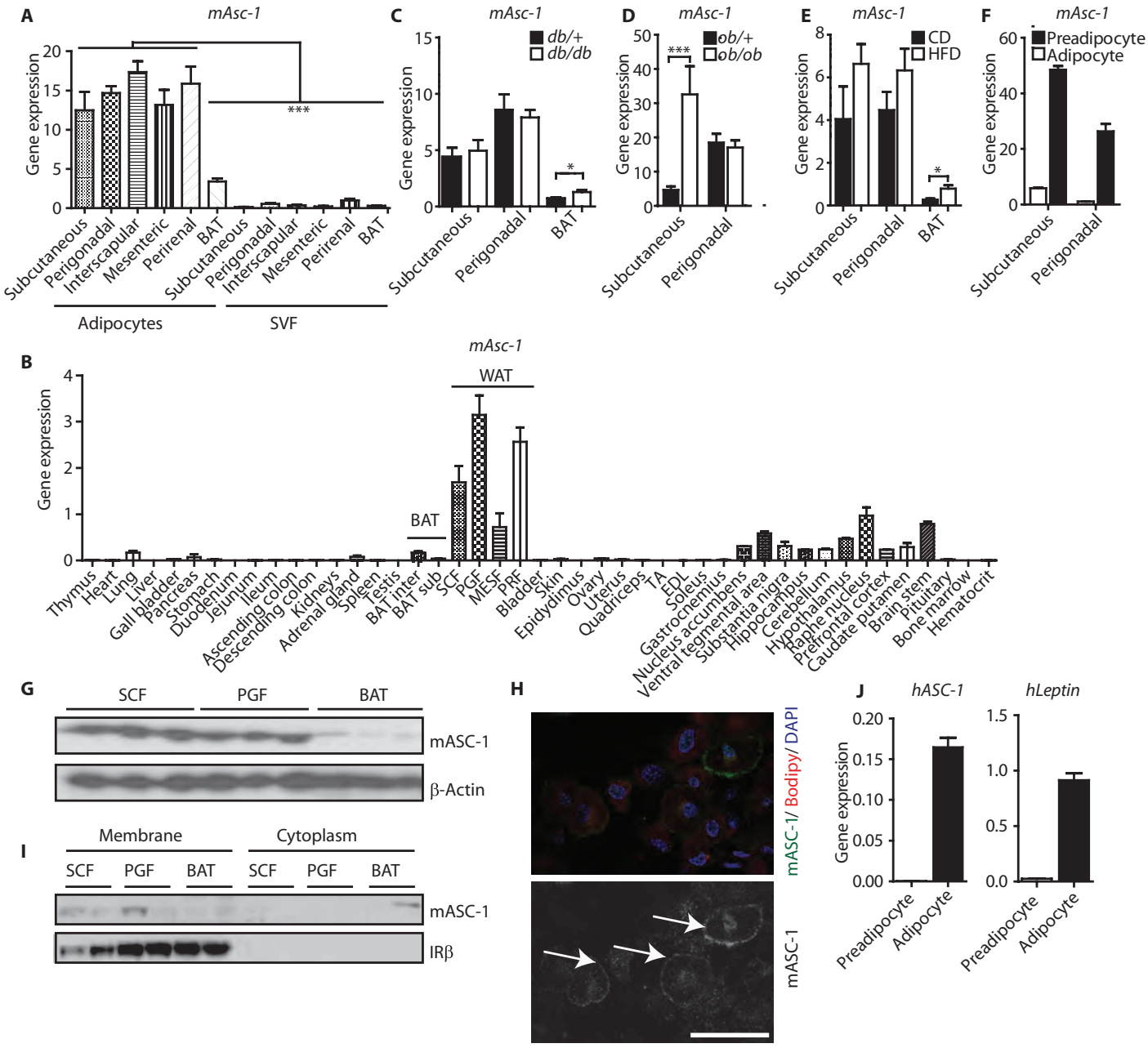


Fig. 1. ASC-1 is a cell surface marker for mouse and human white adipocytes. (**A**) *Asc-1* expression [quantitative polymerase chain reaction (qPCR)] from freshly isolated mouse adipocytes and their corresponding SVF for *Asc-1* from different fat depots ($n = 4$ mice). *$P < 0.05$, one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. (**B**) *Asc-1* expression (qPCR) from 45 tissues from 7-week-old C57BL/6 mice ($n = 3$ mice). Data were normalized to *Tbp*. SCF, subcutaneous white fat; PGF, perigonadal white fat; MESF, mesenteric white fat; PRF, perirenal white fat. (**C**) *Asc-1* expression (qPCR) in subcutaneous, perigonadal, and interscapular BATs of *db/db* and control (*db/+*) mice, normalized to *Tbp* ($n = 6$ mice per group; *$P < 0.05$, two-tailed unpaired *t* test). (**D**) *Asc-1* expression (qPCR) in subcutaneous and perigonadal adipose tissues of *ob/ob* and control (*ob/+*) mice, normalized to *Tbp* ($n = 6$ mice per group; ***$P < 0.001$, two-tailed unpaired *t* test). (**E**) *Asc-1* expression (qPCR) in subcutaneous, perigonadal, and brown adipose tissues of CD- and HFD-fed mice, normalized to *Tbp* ($n = 4$ mice per group; *$P < 0.05$, two-tailed unpaired *t* test). (**F**) *Asc-1* expression in primary murine FACS-sorted preadipocytes and mature adipocytes from SCF and PGF ($n = 2$ mice). Data in (A) to (F) are means ± SEM. (**G**) Western blot for ASC-1 of mouse SCF, PGF, and BAT. β-Actin was used as loading control. (The membrane was stripped and reused in Figs. 2E and 3E, showing the same loading control.) (**H**) Immunofluorescence staining of ASC-1 of differentiated subcutaneous adipocytes from immortalized mouse SVF. Top panel: Cells were costained with the lipid dye Bodipy and nuclear dye 4′,6-diamidino-2-phenylindole (DAPI). Lower panel: Arrows indicate cell surface staining of ASC-1. Scale bar, 50 μm. (**I**) Western blot of mouse membrane extracts and the corresponding cytoplasmic fractions of SCF, PGF, and BAT. IRβ was used as a marker of plasma membrane isolation. (**J**) Expression of human *ASC-1* and *leptin* in primary preadipocytes and fully differentiated (day 10) adipocytes. Data are means ± SEM ($n = 4$).

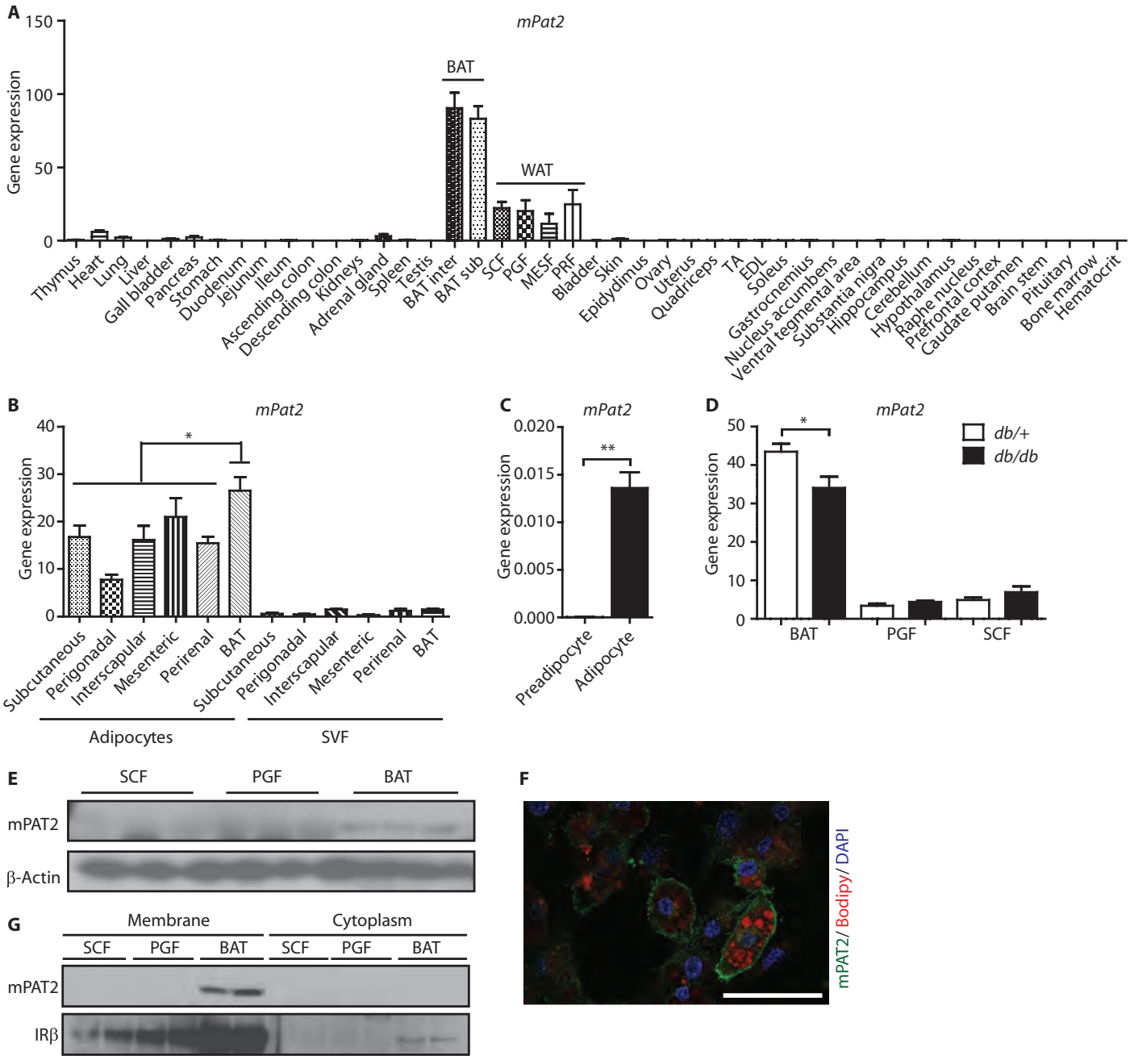


Fig. 2. PAT2 is a cell surface marker of mouse brown adipocytes. (**A**) *Pat2* expression data from 45 tissues of 7-week-old C57BL/6 mice normalized to *Tbp*. Data are means ± SEM (n = 3 mice). (**B**) *Pat2* expression in freshly isolated adipocytes and their corresponding SVF from different mouse fat depots. Data are means ± SEM (n = 4 mice). *P < 0.05, one-way ANOVA with Tukey's multiple comparison test. (**C**) *Pat2* expression in immortalized murine brown preadipocytes and fully differentiated adipocytes. Data are means ± SEM (n = 3 mice; **P < 0.01, two-tailed unpaired t test). (**D**) *Pat2* expression in subcutaneous, perigonadal, and brown adipose tissue of *db/db* and control (*db/+*) mice, normalized to *Tbp*. Data are means ± SEM (n = 6 mice per group; *P < 0.05, two-tailed unpaired t test). (**E**) Western blots for murine PAT2 in SCF, PGF, and BAT. β-Actin was used as loading control. The membrane was stripped and reused in Figs. 1G and 3E, showing the same loading control. (**F**) Immunofluorescence staining of PAT2 of differentiated brown adipocytes from immortalized mouse SVF, costained with the lipid dye Bodipy and nuclear dye DAPI. Scale bar, 50 μm. (**G**) Western blot of membrane extracts and the corresponding cytoplasmic fractions of SCF, PGF, and BAT.

brown adipocytes: *Cd300lg*, tetraspanin 18 (*Tspan18*), frizzled-4 (*Fzd4*), and purinergic receptor P2X, ligand-gated ion channel 5 (*P2rx5*). On further analysis, *Cd300lg*, *Tspan18*, and *Fzd4* expression were not specific (figs. S5 and S6). By contrast, *P2rx5* expression was highest in mouse BAT, with very little expression in WAT. There was also moderate expression in skeletal muscle, heart, brain, adrenal gland, testes, lung, and

skin in mice (Fig. 3A), consistent with previous reports (*22*, *23*). *P2rx5* was expressed at a higher level in brown adipocytes than in mesenteric or perigonadal white adipocytes or the corresponding SVFs (Fig. 3B). Furthermore, *P2rx5* expression was strongly induced upon differentiation of immortalized murine brown preadipocytes (Fig. 3C). Unlike *Asc-1* and *Pat2*, which lost specificity after cell immortalization, *P2rx5* expression remained significantly higher in cultured immortalized brown adipocytes compared to immortalized white adipocytes (fig. S4).

P2rx5 expression in BAT from obese *db*/*db* mice was decreased compared to the lean *db*/+ controls, yet both genotypes were much more highly expressed in BAT than in WAT (Fig. 3D). Likewise, P2RX5 protein was found in brown, but not in white, fat by Western blotting (Fig. 3E). P2RX5 was localized to the cell surface by immunofluorescence staining (Fig. 3F) and membrane fractionation (Fig. 3G), although there was some perinuclear staining, suggesting additional localization in the endoplasmic reticulum.

Regulation of *Asc-1*, *Pat2*, and *P2rx5* during chronic cold exposure and β3-adrenergic stimulation in mice

As demonstrated above, ASC-1 is a white adipocyte–specific surface protein with very little expression in brown adipocytes, whereas PAT2 and P2RX5 are preferentially expressed in brown adipocytes. To study the changes in expression of these markers during "browning" of WAT—that is, the formation of beige fat—we exposed 8-week-old male mice to 5°C for 2 weeks and compared their gene expression to mice housed at 22° to 24°C (Fig. 4A). *Leptin* expression decreased in perigonadal and subcutaneous WAT upon chronic cold exposure and was very low in BAT. *Leptin* was not detectable in hypothalamus and skeletal muscle (tibialis anterior). *Asc-1* mirrored this pattern with decreased expression in WAT upon chronic cold exposure. *Asc-1* expression in the hypothalamus was very low (comparable to BAT) and unaffected by chronic cold exposure (Fig. 4A).

Ucp-1 expression increased in mouse brown fat and subcutaneous white fat upon cold exposure (Fig. 4A). Low levels of *Ucp-1* were detected in perigonadal fat, but not in hypothalamus and skeletal muscle. Similar to *Ucp-1*, *P2rx5* expression increased significantly in brown fat and subcutaneous white fat upon chronic cold exposure, with low expression in skeletal muscle and perigonadal fat. *Pat2* expression was unaltered upon chronic cold exposure in brown and subcutaneous white fat, mimicking the expression pattern of the beige fat marker *Prdm16* (Fig. 4A). Hence, whereas both *Pat2* and *P2rx5* are markers of brown and beige adipocytes, *Pat2* appears to have more similarities to markers of beige fat as opposed to classical brown fat.

To confirm these observations in an independent model of brown adipocyte induction, we treated 4-month-old male mice with daily injections of saline or the β3-adrenergic receptor agonist CL316243 (1 mg/kg) for 2 weeks, inducing the differentiation of beige adipocytes in WAT depots. *Asc-1* expression was significantly higher in perigonadal WAT than in subcutaneous WAT ($P = 0.0003$, two-tailed unpaired t test), and almost undetectable in BAT, with no significant changes upon CL316243 treatment (Fig. 4B). This expression pattern mirrored *leptin* expression (Fig. 4B), although *leptin* mRNA levels increased significantly in BAT with CL316243 treatment. A similar expression pattern was observed for the mouse white adipocyte markers *Hoxc8* and *Hoxc9* (fig. S7A) (*24*).

Ucp-1, a marker of brown fat, was highly expressed in BAT of saline-treated mice, and further increased upon treatment with CL316243. The levels of *Ucp-1* expression were also markedly increased in perigonadal (~6000-fold) and subcutaneous fat (~100-fold) upon β3 stimulation, indicating formation of beige/brown adipocytes within these depots (Fig. 4B). This was confirmed by the presence of UCP-1–positive multilocular adipocytes in both WAT depots of CL316243-treated mice (Fig. 4C). The BAT cell surface markers *Pat2* and *P2rx5* also increased three- to eightfold in subcutaneous and perigonadal fat upon chronic treatment with CL316243 (Fig. 4B).

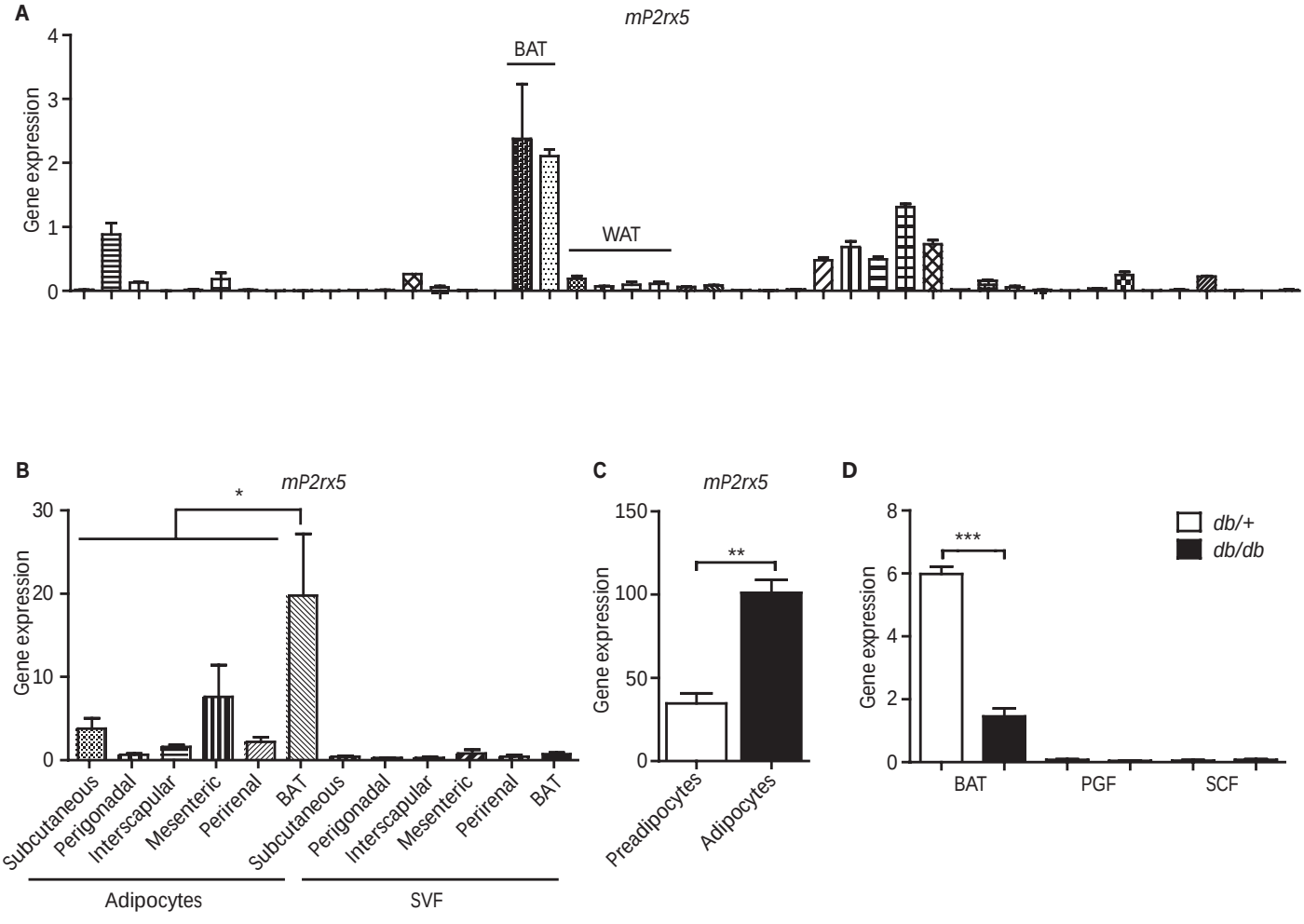
In contrast, the expression of the recently described beige adipocyte markers *Tbx1* and *Tmem26* and the brown adipocyte marker *Prdm16* (*7*) were not significantly changed upon chronic cold exposure or CL316243 treatment (Fig. 4, A and B, and fig. S7). Thus, *Pat2* expression was similar to *Prdm16* in cold-exposed mice, but more similar to *Ucp-1* and *P2rx5* in CL316243-treated mice, suggesting pathways in addition to adrenergic signaling contributing to the response to cold exposure.

ASC-1, *PAT2*, and *P2RX5* in human brown and white fat

To study the expression of *ASC-1*, *PAT2*, and *P2RX5* in human adipose tissue, biopsies from three cohorts of patients were studied. In the first cohort, we obtained biopsies from subcutaneous, carotid sheath, and longus colli fat depots from five patients undergoing neck surgery. Previously reported (*15*) histological analysis and gene expression studies on these samples indicated that markers of BAT were highest in the deeper fat (the longus colli depot) and lowest in the superficial depot, whereas markers of white fat showed the opposite pattern. In agreement, we found that *UCP-1* was more highly expressed in the longus colli fat, followed by carotid sheath fat, and almost undetectable in subcutaneous fat (Fig. 5A). This was paralleled by the expression of *P2RX5* and *PAT2*, which were high in both of the deeper fat depots and very low in subcutaneous fat (Fig. 5A). Conversely, *leptin* expression was highest in subcutaneous fat biopsies and lowest in the deeper depots. Similar expression was observed for *ASC-1* in all three regions, indicating that human BAT is a mixture of brown and white adipocytes.

To further explore the relationship of *ASC-1*, *PAT2*, and *P2RX5*, we performed a comparison to 12 other known markers of adipose cell lineages. For this comparison, we used a total of 24 biopsies from 10 patients from both superficial (subcutaneous; $n = 10$) and deep (around the carotid sheath, longus colli, or prevertebral areas; $n = 14$) fat depots, as described previously (*15*). For this analysis, each individual's deep, brown-enriched fat biopsy was matched with the corresponding superficial (mainly white) fat from the same patient, resulting in 14 paired comparisons. Hierarchical cluster analysis of the deep-to-superficial ratio of expression for each gene identified two major clusters (Fig. 5B). The first cluster of genes included *leptin*, *EBF3*, *FBXO31*, *EVA1*, *CD137*, *TMEM26*, *SHOX2*, *HOXC9*, and *ASC-1* and exhibited low expression in deep versus superficial fat depots, indicative of white adipocytes. The second cluster, reflecting the associations with brown fat, included *UCP-1*, *TBX1*, *ZIC1*, and *LHX8*, as well as *P2RX5* and *PAT2*.

Finally, to gain further insights into the regulation of these surface markers in human WAT, we compared the expression of *ASC-1*, *PAT2*, and *P2RX5* from a third cohort of subjects. These biopsies were obtained from subcutaneous abdominal and intra-abdominal omental fat of lean and obese subjects as previously described (*25*). Similar to the murine expression pattern, *P2RX5* and *PAT2* were barely detectable or very low in whole tissue or isolated adipocytes from either of these white adipose depots (Fig. 5, C and D). In contrast, *ASC-1* was robustly expressed in all WAT samples, with significantly higher levels in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SCF
PGF
BAT
mP2RX5
β-Actin
F
mP2RX5/Bodipy/DAPI
G
mP2RX5
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omental fat compared to subcutaneous fat of obese subjects. Likewise, isolated primary adipocytes from omental fat of obese subjects showed a twofold higher expression of *ASC-1* than subcutaneous adipocytes, with very low levels of expression in the corresponding SVF (Fig. 5D). In lean subjects, there was no significant difference between subcutaneous and omental fat expression of *ASC-1* (Fig. 5C).

DISCUSSION

Adipocytes forming white, brown, and beige adipose tissue depots differ in their developmental origin and gene expression profile (*26*). Thus, white adipocytes have high levels of expression of *leptin* and *adiponectin*, whereas brown adipocytes express high levels of *Ucp-1*, *Zic1*, and *Lhx8*, and beige adipocytes express *Tbx1*, *Tmem26*, and *Tnsfrsf9*, in addition to *Ucp-1*, although the specificity of some of these markers is still debated (*27*). However, the ability to use these markers to separate individual cell types in vitro or in vivo is limited for several reasons. First, most of these markers are either secreted or intracellular proteins, and many are not exclusive to one adipocyte subtype. Some even show high levels of expression in non-adipose tissues. Furthermore, adipose depots can be heterogeneous containing different types of adipocytes, which can change over time or upon physiological, pathophysiological, or pharmacological stimulation. Hence, to understand the nature and function of adipose depots and to translate the beneficial effects of increasing brown and/or beige adipose tissue mass and function into clinical practice, it is essential to find appropriate markers to distinguish these cell types in vivo.

The goal of this study was to identify novel white adipocyte– and brown adipocyte–specific cell surface markers that could be used to mark and target these cells in vitro and in vivo. Using a gene expression database, we identified several surface proteins that are coordinately expressed with *adiponectin* (a marker of white adipocytes) or *Ucp-1* (a marker of brown/beige adipocytes). Although several of these genes had a broader pattern of expression than initially anticipated or were not adipocyte-specific, three markers had the characteristics that were sought: *Asc-1*, *Pat2*, and *P2rx5*.

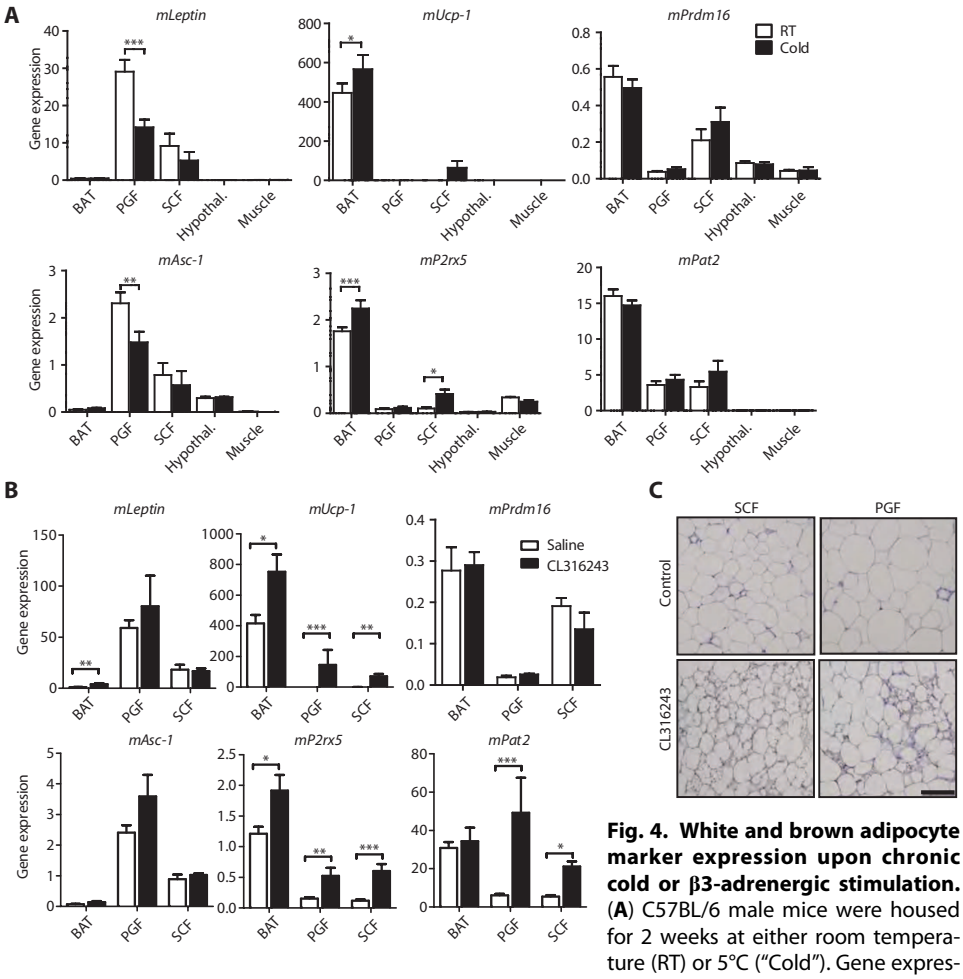

Fig. 4. White and brown adipocyte marker expression upon chronic cold or β3-adrenergic stimulation. (**A**) C57BL/6 male mice were housed for 2 weeks at either room temperature (RT) or 5°C ("Cold"). Gene expression (qPCR) was obtained from BAT, PGF, SCF, hypothalamus, and muscle (tibialis anterior). Data are mean expression normalized to *Tbp* ± SEM (n = 6 mice per group). (**B**) Gene expression (qPCR) in BAT, PGF, and SCF from saline- or CL316243-treated mice. Data are mean expression normalized to *Tbp* ± SEM (n = 7 mice per group). In (A) and (B), *P < 0.05, **P < 0.01, ***P < 0.001, Mann-Whitney or two-tailed unpaired t test, where appropriate. (**C**) α-UCP-1 immunohistochemistry staining of PGF and SCF from saline- or CL316243-treated mice. Scale bar, 100 μm.

Asc-1 is highly enriched in white adipocytes, with very low expression in the SVF and most other peripheral tissues. *Asc-1* expression is also very low in white preadipocytes, but strongly induced upon differentiation, indicating that *Asc-1* is a marker of mature adipocytes. Among WAT depots, *Asc-1* expression is higher in visceral adipose tissue than in subcutaneous fat in both mice and humans. These differences are also observed in freshly isolated human subcutaneous and omental adipocytes, and appear to reflect intrinsic differences in adipose tissue depots. *Asc-1* expression is up-regulated in subcutaneous WAT of leptin-deficient *ob/ob* mice, but not in leptin receptor–deficient *db/db* mice or HFD-fed mice. Thus, it will be important to determine the regulatory elements modulating *Asc-1* expression, especially in obesity.

ASC-1, also known as solute carrier family 7 member 10, is encoded by the *SLC7A10* gene and is a plasma membrane–localized, sodium-independent, neutral amino acid exchange transporter. In the brain, *ASC-1* has high affinity for D-serine, an activator of glutamate/*N*-methyl-D-aspartate receptors (*19*, *20*, *28*). Mice deficient for *Asc-1* show tremors, seizures, and reduced body weight, leading to early death (*29*). Although the function of *Asc-1* in adipose tissue needs further study, in contrast to *leptin* and *adiponectin* (the most commonly used markers of WAT), ASC-1 is not a secreted hormone, but a protein that marks the surface of white adipocytes. In addition, *Asc-1* shows a greater specificity for WAT versus BAT than either *leptin* or *adiponectin*. This

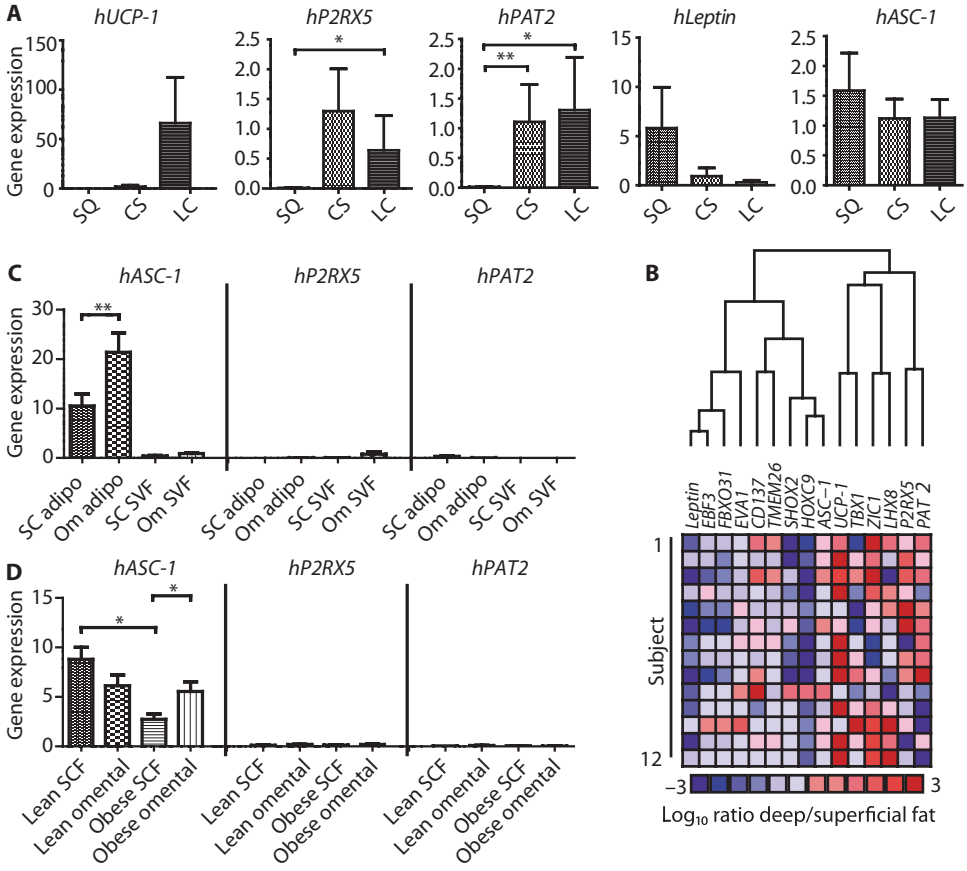

Fig. 5. Expression of *ASC-1*, *PAT2*, and *P2RX5* in human neck fat biopsies. (**A**) qPCR normalized to *TBP* of fat biopsies from subcutaneous (SQ), carotid sheath (CS), and the longus colli (LC) areas ($n = 5$). Statistical significance was determined by Mann-Whitney test. (**B**) Cluster analysis of the $\log_{10}$ ratio of gene expression of deep tissues (BAT) to that of superficial (WAT) fat for previously described white, beige, and brown adipose tissue markers, as well as *ASC-1*, *PAT2*, and *P2RX5* ($n = 14$). (**C**) qPCR for *ASC-1*, *PAT2*, and *P2RX5* from isolated subcutaneous (SC) and omental (Om) adipocytes and SVF of obese subjects ($n = 10$). Target mRNA levels were normalized to the level of *TBP* mRNA, presented as means ± SEM. (**D**) qPCR for *ASC-1*, *PAT2*, and *P2RX5* of whole subcutaneous (SCF) and omental adipose tissue from obese ($n = 13$ and $n = 12$) and lean ($n = 37$ and $n = 12$) subjects.

specificity is lost in immortalized brown adipocytes, demonstrating that immortalization can modify the program of normal gene expression observed in vivo and highlighting the necessity of screens with primary adipocytes or in vivo screens to identify cell type–specific markers. The relatively high levels and variance of ASC-1, as well as *leptin* expression in human deep neck fat depots, highlight the heterogeneity of these BAT depots and underscore the necessity to further characterize these depots before its therapeutical potential can be translated into clinical practice.

In addition to ASC-1 as a marker of white adipocytes, we identified two new cell surface markers of brown/beige adipocytes: PAT2 and P2RX5. PAT2 is part of a four-member family of proton-coupled amino acid transporters, with high affinity for proline, glycine, alanine, and hydroxyproline (*30*, *31*). PAT2 showed the highest specificity for adipose tissue among all three markers identified in this study and could be detected in both white and brown adipocytes, although with significantly higher expression in brown fat. Brown preadipocytes expressed very low levels of *Pat2*, but expression was highly induced upon differentiation, similar to the behavior of *Asc-1* in white preadipocytes.

P2RX5 is part of a seven-member family of adenosine triphosphate–gated ion channels (*30*, *31*), with highest expression in brown adipocytes and BAT. Unlike *Pat2*, the expression of *P2rx5* was very low in white fat, making it more brown fat–specific, although its expression could be detected in skeletal muscle and heart. In contrast to the other two cell surface markers, *P2rx5* was also expressed in brown preadipocytes, and its expression was further increased upon differentiation. As with *Asc-1*, some of the specificity of *Pat2* and *P2rx5* was lost in adipocytes derived from immortalized white and brown preadipocytes, although the expression of *P2rx5* remained significantly higher in brown versus white adipocytes. These data show that although certain characteristics of white and brown adipocytes persist upon prolonged culture or after immortalization, other differences can be lost or diminished. Thus, great care needs to be taken in the interpretation of white versus brown or beige markers without in vivo validation.

Although both PAT2 and P2RX5 are brown fat–specific, they differed in their response to cold exposure and β3-adrenergic stimulation. *P2rx5* expression increased in BAT and subcutaneous white fat after both stimuli and also increased in perigonadal white fat upon CL316243 treatment. This mimicked the response of *Ucp-1* in these same fat depots. In contrast, *Pat2* did not change its expression in any fat depot upon chronic cold exposure, but was strongly increased in subcutaneous and perigonadal white fat to levels equal to that of BAT in CL316243-treated mice. These data suggest that PAT2 and P2RX5 mark distinct brown/beige adipocyte populations. This is in line with studies suggesting that mixtures of beige and brown adipocytes may coexist within white adipose depots (*7*, *15*, *16*).

Several human genes have been described as being useful to differentiate between constitutive brown and inducible beige adipocytes. However, most of these genes, including *TBX1*, *HOXC8*, *HOXC9*, *ZIC1*, and *LHX8*, encode nuclear proteins, and therefore not easily accessible for targeting in vivo. In contrast, *PAT2* and *P2RX5*, as well as the previously described *TMEM26* and *TNFRSF9*, encode cell surface proteins. These latter genes were identified by comparison of WAT and BAT or selecting single-cell preadipocyte clones from white fat pads (*27*). Hence, by the nature of the experimental design, there was limited analysis of non-adipose tissues to determine whether these will provide useful markers to target brown fat in vivo. In contrast, PAT2 and P2RX5 fulfill all requirements to be used for targeting for drug delivery to brown/beige fat in vivo as they are localized on the cell surface and show a very high specificity for brown adipocytes compared to more than 40 other tissues.

Analysis of human neck fat biopsies confirmed the expression of *P2RX5* and *PAT2* in human BAT. *P2RX5* and *PAT2* were highly expressed in deep cervical fat depots (fat near the carotid sheath or longus colli muscle), which are known to contain cells with high *UCP-1* expression (*15*). In the current study samples, we found that *UCP-1* expression was higher in longus colli fat than in carotid sheath fat. Because the expression of *UCP-1* reflects the amount of brown fat as well as its state of activity (*32*), the relatively lower levels of *UCP-1* with high levels of *P2RX5* and *PAT2* in the carotid sheath fat may indicate the presence of inactive or less active brown fat in these areas. Hence, the markers described here could be used in future experiments to dissect the heterogeneity and activity state of individual human BAT depots by further detailing the nature of UCP-1–, P2RX5-, PAT2-, and ASC-1–positive adipocytes.

To date, identification and classification of brown fat in humans relies mainly on positron emission tomography scanning techniques using [^{18}F]fluorodeoxyglucose uptake into metabolically active brown adipocytes. Although there are some reports indicating that magnetic resonance imaging can also assess brown fat in humans (*33*), most current imaging methods do not allow quantification of inactive brown fat, which is essential if one is to determine total response of brown fat mass to any physiological alterations or pharmacological stimulation. Thus, experiments directed at finding appropriate antibodies or other targeting molecules to extracellular epitopes of ASC-1, PAT2, and P2RX5 should allow translation of our findings to both quantitative imaging and targeting of drugs in vivo to human brown and white fat.

In conclusion, the cell surface proteins ASC-1, PAT2, and P2RX5 provide novel tools to selectively mark and access intact white and brown adipocytes in vivo and in primary isolated cells in vitro. These markers should be useful in defining the heterogeneity and intrinsic differences of adipose tissue depots, and agents directed against them will enable the translation of our findings for diagnostic and therapeutic purposes.

MATERIALS AND METHODS

Study design

The objective of this study was to identify novel white and brown adipocytes cell surface markers in mice and validate their specificity in human fat tissue biopsies. To identify potential candidate genes, we queried the murine expression database (http://www.biogps.org) to identify surface proteins that correlated to the gene expression pattern of the white adipocyte marker *adiponectin* and the brown adipocyte marker *Ucp-1*. Candidate genes were tested by qPCR on multiple tissues of wild-type C57BL/6 mice, and their expression pattern was studied in genetically obese *ob/ob* and *db/db* mice, as well as in mice made obese by feeding an HFD and mice stimulated to activate brown fat and induce browning of WAT, namely, chronic cold exposure and treatment with the β3-adrenergic receptor agonist CL316243. White and brown adipocyte specificity and membrane localization were validated by Western blot and immunofluorescence staining of selected candidate genes. These observations in mice were translated to humans by using white and brown fat biopsies from three previously published (*15*, *25*) patient cohorts.

Mice

All protocols were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and in accordance with the National Institutes of Health (NIH) guidelines. Mice (The Jackson Laboratory) were maintained on a 12-hour light/dark cycle and fed a normal CD (9F5020; PharmaServ). To induce browning of white fat, 8-week-old C57BL/6 male mice were housed at 5°C in a thermocontrolled incubator under the same diet and light conditions for 2 weeks. In addition, 4-month-old male mice on a mixed C57BL/6 and 129Sv background were injected intraperitoneally with either saline or CL316243 (1 μg/g) daily for 14 days, as described previously (*3*). To study the effects of HFD (D12492)–induced expression changes, 4-month-old male mice on a mixed C57BL/6 and 129Sv background were fed a HFD diet containing 60% calories from fat for 8 weeks. For tissue expression studies, 7-week-old C57BL/6 mice were anesthetized and perfused with PBS using intracardiac perfusion before tissue harvest. Tissues from 12-week-old male C57BL/6 *db/db*, *db/+*, *ob/ob*, and *ob/+* mice were also collected, but without intracardiac perfusion.

Human study population

Human brown fat and corresponding superficial white fat biopsies were obtained during anterior cervical spine surgery or thyroidectomy (n = 24 biopsies from 10 patients). Written informed consent was obtained before surgery (*15*). All patients undergoing thyroidectomies had thyroid-stimulating hormone values within the normal range. This study was approved by the Human Studies Institutional Review Boards of Beth Israel Deaconess Medical Center and Joslin Diabetes Center.

In additional cohorts, human fat biopsies were obtained from the abdominal region of lean and obese subjects, with written informed consent. The study was approved by the regional committee for Medical and Health Research Ethics, Western Norway (REK Vest). Expression was compared between whole subcutaneous and omental fat from lean and obese subjects, and between isolated subcutaneous and omental adipocytes and SVF from obese subjects. An overview of the cohorts is shown in table S1. In some studies, whole tissue was collected by surgical excision during bariatric surgery (obese subjects) or elective surgery (lean subjects) and immediately placed in liquid nitrogen. Adipocytes and SVF were separated as described previously (*25*). Briefly, about 700 to 800 mg of tissue were fractionated by collagenase for 30 min at 37°C immediately after excision. The released cells were sieved through a 200-μm polypropylene filter. Floating cells (adipocytes) were transferred to a 2-ml tube, washed in 1 ml of Hanks' balanced salt solution with 5% bovine serum albumin, and collected after floating. The nonfloating cells (SVF) were centrifuged at 2500 rpm for 5 min, and the supernatant was removed. The adipocytes and SVF were lysed in QIAzol Lysing Reagent (Qiagen) and frozen in liquid nitrogen.

Adipocyte marker identification

The expression pattern of mouse *adiponectin* and *Ucp-1* was used to search the SymAtlas database (http://www.biogps.org) for genes with correlated (coefficient >0.95) expression pattern. These candidate genes were filtered for high expression in fat (at least three times the mean of all other tissues) with less than the mean levels of expression in skeletal muscle, heart, lung, pancreas, cerebellum, and cerebral cortex. Identified candidates were used for further validation.

Statistical analysis

Data are presented as means ± SEM. All statistical analysis was performed using GraphPad Prism. Normal distribution of samples was tested, where appropriate, to select parametric or nonparametric tests as indicated in the figure legends. Two-tailed Student's t test or one- or two-way ANOVA for multiple comparisons was used to determine P values, unless indicated differently in the figure legend.

SUPPLEMENTARY MATERIALS

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Methods
Fig. S1. Identification of adipocyte-specific surface proteins in mice.
Fig. S2. Expression of *adiponectin*- and *Nrg4*-correlated genes in mice.
Fig. S3. *Adiponectin*- and *Nrg4*-correlated genes are specific for adipocytes in mice.
Fig. S4. Expression of *Asc-1*, *PAT2*, and *P2RX5* in differentiated and undifferentiated immortalized mouse SVF.
Fig. S5. Tissue expression of *UCP-1*– and *PAT2*-correlated genes.
Fig. S6. Adipocyte specificity of *UCP-1*– and *PAT2*-correlated genes.
Fig. S7. Expression of *Hoxc8*, *Hoxc9*, *Tbx1*, and *Tmem26* upon chronic cold and β3-adrenergic stimulation.
Table S1. Overview of the human abdominal adipose tissue samples from lean and obese subjects.
Table S2. Mouse and human qPCR primer sequences.
Reference (*34*)

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