Intrinsic Heterogeneity in Adipose Tissue of Fat-specific Insulin Receptor Knock-out Mice Is Associated with Differences in Patterns of Gene Expression

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Mice with a fat-specific insulin receptor knock-out (FIRKO) have reduced adipose tissue mass, are protected against obesity, and have an extended life span. White adipose tissue of FIRKO mice is also characterized by a polarization into two major populations of adipocytes, one small (<50 μm) and one large (>100 μm), which differ with regard to basal triglyceride synthesis and lipolysis, as well as in the expression of fatty acid synthase, sterol regulatory element-binding protein 1c, and CCAAT/enhancer-binding protein α (C/EBP-α).

Gene expression analysis using RNA isolated from large and small adipocytes of FIRKO and control (IR lox/lox) mice was performed on oligonucleotide microarrays. Of the 12,488 genes/expressed sequence tags represented, 63 genes were expressed differentially in the four populations of adipocytes at the p < 0.001 level. These alterations exhibited 10 fold changes and occurred in response to two distinct regulatory effects. 63 genes were identified as changed in expression depending primarily upon adipocyte size, including C/EBP-α, C/EBP-β, superoxide dismutase 3, and the platelet-derived growth factor receptor. 48 genes were regulated primarily by impairment of insulin signaling, including transforming growth factor β, interferon γ, insulin-like growth factor I receptor, activating transcription factor 3, aldehyde dehydrogenase 2, and protein kinase Cα. These data suggest an intrinsic heterogeneity of adipocytes with differences in gene expression related to adipocyte size and insulin signaling.

Adipocytes play a central role in energy balance, both as a reservoir, storing and releasing fuel, and as endocrine cells, secreting factors that regulate whole body energy metabolism (1). The loss of insulin action selectively in adipose tissue in insulin receptor knock-out fat of the FIRKO mice unmasks a heterogeneity of WAT.

leads to profound changes in cellular function, including changes in glucose metabolism, lipid storage, and protein expression (2). We reported previously that FIRKO mice have markedly reduced fat mass and whole body triglyceride stores and are protected from gold thioglucose-induced and age-related obesity, as well as the associated glucose intolerance (2). These changes are associated with increased longevity of these mice (3). FIRKO mice also exhibit a heterogeneity in fat cell size with polarization into small (diameter <50 μm) and large (diameter >100 μm) subclasses of adipocytes (2). This polarization by cell size of adipose tissue is not unique to the FIRKO model. Targeted disruption of the hormone-sensitive lipase also causes reduced body fat mass and heterogeneity of adipocytes in white adipose tissue (WAT) with a population of larger adipocytes, which is associated with lipid accumulation, and a population of small adipocytes, which is not (4, 5). These results suggest that adipocytes, even within a single depot of WAT, may not be of a single lineage or type (6).

Using a candidate gene/protein approach we found that the heterogeneity of WAT in FIRKO mice is accompanied by changes in the expression of several key adipocyte proteins, such as ACRP30, fatty acid synthase, SREBP-1 C/EBP-α, and GLUT-1 glucose transporter (2). These results led to our hypothesis that insulin receptor knock-out unmasks a naturally occurring heterogeneity of adipocytes with differential roles in lipid storage and other functions. Recently, genomewide expression profiling has been used to investigate adipogenesis and adipocyte gene expression in vitro and in vivo (7, 8). However, until now there has been no study attempting to define the full set of alterations in gene expression, which may contribute to heterogeneity in WAT.

In the present study, we have used a genomewide expression profiling approach to test the hypothesis that the insulin receptor knock-out fat of the FIRKO mice unmasks a heterogeneity of adipocytes that can be defined by subsets of small and large adipocytes. We have further addressed the question as to which genes are primarily affected by the absence or presence of insulin signaling and which are regulated by the adipocyte size. In addition, because protein expression may uncover additional changes that result from post-transcriptional effects (9), we further characterized the protein expression pattern in these large and small adipocytes from FIRKO and control mice.
using a global proteomics approach and compared that with the gene expression pattern (10).

MATERIALS AND METHODS

Animals—IR (lox/lox) mice were derived as described previously (11) and maintained on a mixed (C57BL/6 × 129Sv) genetic background. Adipose tissue or FIRKO mice were derived by crossing double heterozygous IR (lox/+) with IR (lox/+) mice that also expressed Cre recombinase under the control of the fatty acid-binding protein aP2 promoter/enhancer (aP2-Cre-IR(lox/+)) as described previously (2). Animals for this study were derived from the breedings of IR lox/lox with F1 mice. Adipocyte size was determined by dividing the lipid content of the cell suspension by the cell number (12) and in addition by a Multisizer (Beckman-Coulter) as described previously (13).

Glucose Transport and Metabolism in Isolated Adipocytes—For the determination of glucose transport, isolated adipocytes of different diameter were stimulated with 100 nM insulin for 30 min and then incubated for 30 min with 3 μM U-14Cglucose (14). Immediately after the incubation adipocytes were fixed with osmic acid, incubated for 48 h at 37 °C (15), and the radioactivity was quantified. Glucose metabolism into three different pathways was determined at 5 mM glucose (containing 3 μM U-14Cglucose) using a 10% isolated fat cell suspension. Glucose incorporated into triglycerides and CO₂ was measured after a 1-h incubation in the absence or presence of 80 nM insulin as described previously (13). Glucose incorporated into lactate was measured after a 1-h incubation and after separation of lactate from glucose using union exchange columns (AG 1-X8 Resin, Bio-Rad).

Sample Preparation, Microarray, and Data Analysis—Equal quantities of total RNA were isolated from isolated pooled small and large adipocytes from at least 9–12 F1 mice and at least 8–10 IR lox/lox mice (see Fig. 1). Double-stranded cDNA synthesis was reverse transcribed from 15 μg of isolated mRNA by using the SuperScript choice system (Invitrogen) in addition to using an oligo(dT) primer containing a T7 RNA polymerase promoter site. Double-stranded cDNA was purified with Phase Lock Gel (Eppendorf). Biotin-labeled cRNA was transcribed from cDNA using a BioArray RNA transcript labeling kit (Enzo). A hybridization mixture containing 15 μg of biotinylated cRNA, adjusted for possible carryover of residual total RNA, was prepared and hybridized to mouse Affymetrix MG-U74A-v2 chips.

Isolation of Adipocytes and Separation into Small and Large Adipo- cytes—At the age of 3 months, male animals were sacrificed, and epidydimal fat pads were removed. Adipocytes were isolated by collagenase digestion. Separation of cells into small and large diameter adipocytes was achieved by filtering the adipocyte suspension through a 75-μm pore size nylon mesh screen (see Fig. 1). Aliquots of adipocytes were fixed with osmic acid and counted in a Coulter counter (12). Adipocyte size was determined by dividing the lipid content of the cell suspension by the cell number (12) and in addition by a Multisizer (Beckman-Coulter) as described previously (13). Glucose incorporated into lactate was measured after a 1-h incubation and after separation of lactate from glucose using union exchange columns (AG 1-X8 Resin, Bio-Rad).

Histology—Tissues were fixed in 10% buffered formalin and imbedded in paraffin. Multiple sections (separated by 70–80 μm each) were obtained from gonadal fat pads and analyzed systematically with respect to adipocyte size and number. Staining of the sections was performed with hematoxylin and eosin. For each genotype and gender at least 10 fields (representing 100 adipocytes/field) were analyzed. Images were acquired using BX60 microscope (Olympus) and a HV-C20 television camera (Hitachi, Japan) and were analyzed using Image-Pro Plus 4.0 software.

Isolation of Adipocytes and Separation into Small and Large Adipocytes—At the age of 3 months, male animals were sacrificed, and epidydimal fat pads were removed. Adipocytes were isolated by collagenase digestion. Separation of cells into small and large diameter adipocytes was achieved by filtering the adipocyte suspension through a 75-μm pore size nylon mesh screen (see Fig. 1). Aliquots of adipocytes were fixed with osmic acid and counted in a Coulter counter (12). Adipocyte size was determined by dividing the lipid content of the cell suspension by the cell number (12) and in addition by a Multisizer (Beckman-Coulter) as described previously (13).

Quantitative Reverse Transcription Real Time PCR—Total RNA from each of the same 20 pooled RNA samples, 5 each from the 4 experimental groups that were hybridized to the microarrays, was used as a template in a fluorescence-based, quantitative one-step real time PCR, with specific primers for selected genes (n = 15) to confirm the microarray results.

RESULTS

We have previously shown that adipose-specific insulin receptor knock-out causes a change in the morphology of WAT with a polarization into small (diameter <50 μm) and large (diameter >100 μm) subclasses of adipocytes (2). Histological sections representative of WAT in FIRKO and IR lox/lox mice demonstrate a relatively uniform and normally distributed adipocyte size in WAT from IR lox/lox mice, whereas the fat pads
From FIRKO mice contain a mixed population of the two cell sizes (2). Western blot analysis of candidate molecules indicates near complete knock-out of the insulin receptor in both classes of adipocytes, but the polarization of adipocytes is accompanied by differences between small and large adipocytes in the expression of several key adipocyte proteins including ACRP30, fatty acid synthase, glucose transporter GLUT 1, SREBP 1c, and C/EBP-α (2). In addition, as reported previously (2), in control mice, insulin-stimulated glucose uptake was significantly higher in small adipocytes (about 5-fold stimulation) compared with large adipocytes (about 3-fold stimulation), consistent with a decrease in insulin responsiveness in larger adipocytes. In adipocytes from FIRKO mice, basal glucose uptake was unchanged compared with the controls, but insulin-stimulated glucose uptake was reduced by more than 90% in both large and small cells, confirming the efficiency of the IR knock-out. On the other hand, basal incorporation of glucose into triglycerides and basal rates of lipolysis in large FIRKO adipocytes were increased significantly compared with the other groups (p < 0.05) (2), indicating increased lipid turnover in large adipocytes of FIRKO mice. As expected, insulin inhibited lipolysis in both large and small cells of control mice, but not in the FIRKO mice (2).

To further analyze the differences in adipocyte populations, the experimental protocol shown in Fig. 1 was applied. To assure uniformity of recombination of the insulin receptor allele in the FIRKO study groups, IR recombination was assessed in WAT of each mouse by a PCR technique described previously (2), and only data from mice with an efficient IR recombination were included in the analysis. The three littersmate control groups (wild type, IR (lox/lox), and aP2-Cre mice) were indistinguishable with regard to physiologic and metabolic parameters and possessed the same mixed genetic background as the FIRKO mice, including C57BL/6J, 129Sv, and FVB. To characterize the gene expression using the genomics approach, adipocytes were isolated from epididymal fat pads from 3-month-old male FIRKO and IR lox/lox mice and separated into small (diameter <75 μm) and large (diameter >75 μm) by filtration through nylon mesh screen as described in detail under “Materials and Methods” (Fig. 1). For all genomic and proteomic studies, IR lox/lox mice were used as controls.

**Microarray Analysis**—Gene expression analysis was conducted using Affymetrix microarrays and subjected to three filters of significance. The first filter excluded all genes that had a mean expression value that was below the sum of the average background and the average standard difference threshold (equal to 4 times the scaled noise) in all groups. 7,389 genes passed the first significance filter, which was to consider only those genes for which the difference between the means of the compared experimental groups was >2 times the sum of the standard differences of both groups. Finally, Student’s t test was used as a third filter in each of the four two-way comparisons (FIRKO large (L) versus small (S) adipocytes, IR lox/lox large versus small adipocytes, large adipocytes; FIRKO versus IR lox/lox, small adipocytes; FIRKO versus IR lox/lox). 221 genes that passed all three filters including the significance level of p < 0.0001 and were identified as significantly changed. B, comparisons performed to test for the consequences of the impaired insulin signaling or the adipocyte size.

**FIG. 2. Data analysis. A**, three separate independent filters of significance were applied serially to obtain a list that was significantly up- or down-regulated in at least one comparison. The first filter excluded all genes that had a mean expression value that was below the sum of the average background (BG) and the average standard difference threshold (equal to 4 times the scaled noise) in all groups. 7,389 genes that passed this first filter were subjected to the second significance filter, which was to consider only those genes for which the difference between the means of the compared experimental groups was >2 times the sum of the standard differences of both groups. Finally, Student’s t test was used as a third filter in each of the four two-way comparisons (FIRKO L versus S adipocytes, IR lox/lox L versus S adipocytes, large adipocytes; FIRKO versus IR lox/lox, small adipocytes; FIRKO versus IR lox/lox). 221 genes that passed all three filters including the significance level of p < 0.0001 and were identified as significantly changed. B, comparisons performed to test for the consequences of the impaired insulin signaling or the adipocyte size.

**Table I.**

| Comparisons | Influence of impaired insulin signaling on gene expression | Influence of adipocyte size on gene expression |
|--------------|----------------------------------------------------------|---------------------------------------------|
| Large FIRKO vs. Large IR lox/lox | | |
| Small FIRKO vs. Small IR lox/lox | | |
| FIRKO large vs. FIRKO small | | |
| IR lox/lox large vs. IR lox/lox small | | |

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| IR lox/lox large vs. IR lox/lox small | | |

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| IR lox/lox large vs. IR lox/lox small | | |

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**Gene Expression Reflects Heterogeneity of Adipocytes**

**Table 1**

Changes in gene expression in adipocytes of FIRKO mice depending on impaired insulin signaling

| Accession no. | Gene Description | Large adipocytes | Small adipocytes |
|---------------|------------------|------------------|------------------|
|               |                  | FIRKO IR lox     | FIRKO IR lox     |
| Metabolism    |                  |                  |                  |
| X68848        | Fructose-6-phosphatase 1 | 0.72 <0.001 0.2 | 0.47 <0.001 |
| X69888        | Hydroxysteroid 17 β-dehydrogenase 4 | 1.35 <0.001 1.2 | 0.34 0.01 |
| M25999        | Glyceraldehyde-3-phosphate dehydrogenase | 0.46 0.1 | 0.3 <0.001 |
| U31966        | Carboxyl reductase 1 | 1.02 0.8 | 0.77 <0.001 |
| U92793        | α-Glucosidase 2 | 1.84 <0.001 1.2 | 0.3 0.03 |
| Immune response/secerted molecules | | | |
| U14513        | IFN-γ | 0.31 <0.001 0.5 | 0.2 0.02 |
| U50712        | Small inducible cytokine A12 | 0.16 <0.001 0.31 | 0.6 <0.001 |
| AF013486      | IFN receptor 2 | 0.99 0.9 | 0.66 <0.001 |
| AA914345      | IFN inducible GTPase | 0.7 <0.001 0.98 | 0.95 0.01 |
| L20315        | Macrophage expressed gene 1 | 0.29 <0.001 0.53 | 0.01 0.01 |
| M57891        | Complement component 2 | 1.78 <0.001 1.4 | 0.06 0.06 |
| U23778        | B-cell leukemia-related protein A1b | 0.38 <0.001 0.7 | 0.2 0.1 |
| U27195        | Leukotriene C4 synthase | 0.61 <0.001 0.6 | 0.03 0.01 |
| X15417        | P-lysozyme structural | 0.19 <0.001 0.26 | 0.01 0.01 |
| X62502        | Small inducible cytokine A4 | 0.27 <0.001 0.66 | 0.3 0.03 |
| D84196        | TNF-α | 1.38 0.09 | 2.24 <0.001 |
| X68861        | Complement component 1q | 0.36 <0.001 0.8 | 0.3 0.03 |
| A1652144      | Pre-B-cell colony-enhancing factor | 0.91 0.6 | 0.66 <0.001 |
| M33960        | PAI-1 | 2.4 <0.001 2.7 | 0.05 <0.001 |
| A1837100      | CD83 antigen | 0.53 <0.001 0.76 | 0.1 0.2 |
| AW125574      | Williams-Beuren syndrome region 5 homolog | 0.43 <0.001 0.61 | 0.03 0.03 |
| M82831        | Matrix metalloproteinase 12 | 0.25 <0.001 0.51 | 0.01 0.01 |
| U58907        | Cystatin B | 0.7 <0.001 0.88 | 0.4 0.01 |
| U16834        | Lectin, galactose binding | 0.46 <0.001 0.77 | 0.1 0.01 |
| X17089        | FK506-binding protein 4 | 1.58 <0.001 1.76 | 0.05 0.05 |
| Mitochondrial molecules | | | |
| U07235        | Aldehyde dehydrogenase 2 | 1.55 <0.001 1.41 | 0.03 0.01 |
| A1535992      | Mitochondrial ribosomal protein S21 | 1.54 <0.001 1.23 | 0.2 0.02 |
| A1849787      | ATP synthase-mitochondrial F1 complex | 0.65 0.02 | 0.66 <0.001 |
| Cytoskeleton, transport, trafficking molecules | | | |
| A9861490      | Procollagen, type VI, α3 | 0.97 0.7 | 1.47 <0.001 |
| AW209098     | IQ motif containing GTPase-activating protein 1 | 0.56 <0.001 0.65 | 0.01 0.01 |
| A1858649      | Dynactin 4 | 0.83 0.01 | 0.7 <0.001 |
| D00472       | Cofilin 1, non-muscle | 0.65 0.01 | 0.73 <0.001 |
| D37837       | Plastin 2, L | 0.38 <0.001 0.67 | 0.1 0.01 |
| M83749       | Cyclin D2 | 0.41 0.07 | 0.32 <0.001 |
| U33196       | Y box protein 1 | 0.96 0.8 | 0.62 <0.001 |
| X54056       | Proprotein convertase subtilisin | 1.5 <0.001 1.29 | 0.05 0.05 |
| Development and proliferation factors | | | |
| U14739       | Four and one-half LIM domains 1 | 1.35 <0.001 1.23 | 0.17 0.01 |
| X96546       | Sialyltransferase B 8 | 3.2 0.07 | 3.74 <0.001 |
| Transcription factors | | | |
| U19118       | Activating transcription factor 3 | 0.56 <0.001 0.86 | 0.35 0.35 |
| A1845517     | Ring finger protein 4 | 0.81 0.06 | 0.69 <0.001 |
| A1845585     | Zinc finger protein 289 | 1.48 <0.001 0.99 | 0.9 0.01 |
| U89489       | LIM domain binding 2 | 0.59 <0.001 1.17 | 0.3 0.3 |
| Ribosomal proteins | | | |
| M14108       | Ribosomal protein S16 | 0.73 <0.001 0.93 | 0.4 0.03 |
| M14689       | Ribosomal protein L7a | 1.36 <0.001 1.34 | 0.08 0.01 |
| X83590       | Rosomol protein L5 | 1.27 0.02 | 1.55 <0.001 |
| Signaling factors | | | |
| Z43524       | Protein kinase C, α | 0.84 0.5 | 0.43 <0.001 |
| A137205       | MAP kinase kinase 1 | 0.73 <0.001 0.81 | 0.16 0.01 |
| A1928421      | Casein kinase 1 | 0.88 0.24 | 0.64 <0.001 |
| AF089606     | KIR 4.2 | 0.34 <0.001 0.37 | 0.02 0.02 |
| A1839296      | LDL receptor-related protein 10 | 1.2 0.1 | 1.35 <0.001 |
| A1849587      | Calcium channel, voltage-dependent | 0.86 0.4 | 0.61 <0.001 |
| L02914       | Aquaporin 1 | 0.28 <0.001 0.91 | 0.7 0.01 |
| U40825       | WW domain binding protein 1 | 0.96 0.7 | 0.67 <0.001 |
| U43512       | Dystroglycan 1 | 1.42 <0.001 1.32 | 0.05 0.05 |
| X61399       | MARCKS-like protein | 0.56 <0.001 0.66 | 0.04 0.01 |
| X78505       | Discoidin domain receptor family 2 | 0.6 <0.001 1.11 | 0.6 0.01 |
| Growth factors | | | |
| M94450       | Growth factor receptor bound protein 7 | 2.06 <0.001 1.32 | 0.5 0.01 |
| M22745       | TGF-β3 | 1.2 0.4 | 1.93 <0.001 |
| Others | | | |
| A0722200     | Methyl-CpG binding domain protein 1 | 2.7 <0.001 1.63 | 0.07 0.01 |
| A093624      | Reticulin 2 | 0.56 <0.001 0.5 | 0.04 0.04 |
| X7853        | Fibulin 1 | 0.66 <0.001 0.92 | 0.01 0.01 |
| AF022232     | Bcl2-associated athanogene 1 | 1.65 0.01 | 1.59 <0.001 |
Gene Expression Reflects Heterogeneity of Adipocytes

### Table II

Changes in gene expression in adipocytes of FIRKO and control mice depending on adipocyte size

| Accession no. | Gene                                                                 | FIRKO adipocytes | IR lox adipocytes |
|--------------|----------------------------------------------------------------------|------------------|------------------|
|              |                                                                      | Large | Small | Large | Small |
| Metabolism   |                                                                      |       |       |       |       |
| AB016780     | Glutamine fructose-6-phosphate                                       | 1.02  | 0.8   | 2.99  | <0.001|
| D93063       | Protein glycosynase                                                  | 1.25  | 0.2   | 1.84  | <0.001|
| L9192        | Pyruvate decarboxylase                                               | 0.78  | 0.5   | 0.47  | <0.001|
| M15268       | Aminolevulinic acid synthase                                         | 1.04  | 0.9   | 3.8   | <0.001|
| U31966       | Carbonyl reductase 1                                                | 0.98  | 0.9   | 0.75  | <0.001|
| AW123952     | LDH 1                                                                | 0.82  | 0.6   | 2.78  | <0.001|
| Immune response/secrated molecules |                                                            |       |       |       |       |
| AF061260     | Immunoglobulin superfamily, member 4                                | 1.22  | 0.06  | 1.75  | <0.001|
| A1123585     | Complement component 1                                              | 1.22  | 0.7   | 1.79  | <0.001|
| AV248632     | Cytokine inducible SH2-containing protein                            | 1.41  | 0.4   | 0.36  | <0.001|
| L15435       | TNF superfamily, member 9 (IL1)                                      | 0.81  | 0.7   | 4.25  | <0.001|
| M13926       | Granulocyte colony stimulating factor 3                              | 1.07  | 0.6   | 2.71  | <0.001|
| M74123       | IFN-activated gene 204                                               | 1.75  | 0.15  | 2.57  | <0.001|
| M82331       | Matrix metalloproteinase 12                                          | 1.41  | 0.33  | 2.9   | <0.001|
| U23781       | B-cell leukemia/lymphoma 2 related protein A1d                      | 1.13  | 0.5   | 2.07  | <0.001|
| X58881       | Complement component 1q                                             | 0.85  | 0.5   | 1.85  | <0.001|
| AF045887     | Angiotensinogen                                                     | 0.82  | <0.08  | 0.76  | <0.001|
| AF083182     | Lyphocyte antigen 57                                               | 0.36  | >0.001 | 0.7  | 0.2   |
| M15131       | IL-1β                                                               | 2.08  | 0.04  | 3.55  | <0.005|
| Mitochondrial | Mitochondrial ABC protein 2                                        | 0.86  | 0.6   | 0.25  | <0.001|
| D58534       | Cytochrome P450 subfamily IV B                                      | 1.24  | 0.06  | 1.62  | <0.001|
| U38261       | Superoxide dismutase 3                                              | 0.83  | 0.4   | 0.78  | <0.001|
| Cytoskeleton, transport, trafficking |                                                            |       |       |       |       |
| AA655199     | Collagen, type III, α                                                | 1.27  | 0.44  | 2.14  | <0.001|
| AB012903     | CD47                                                                 | 1.37  | 0.11  | 1.51  | <0.001|
| U15012       | Growth hormone receptor                                             | 0.73  | 0.008 | 0.69  | 0.001|
| AF013262     | Lumican                                                             | 1.77  | 0.1   | 3.15  | <0.001|
| A1042802     | Taurin                                                              | 1.51  | 0.4   | 0.27  | <0.001|
| A1352649     | Dynactin 4                                                          | 3.13  | <0.001 | 1.1  | 0.09  |
| U319788      | Claudin 5                                                           | 1.07  | 0.7   | 1.77  | <0.001|
| Transcription factors |                                                            |       |       |       |       |
| A1844317     | Ring finger protein 4                                               | 0.95  | 0.66  | 0.81  | <0.001|
| M18169       | Transcription elongation factor A (SII) 1                            | 0.93  | 0.68  | 0.71  | <0.001|
| U47543       | Nrf-A-binding protein 2                                             | 0.89  | 0.64  | 2.62  | <0.001|
| X61800       | C/EBP-β                                                            | 1.4   | 0.05  | 1.94  | <0.001|
| U89489       | LIM domain binding 2                                                | 0.92  | 0.54  | 1.83  | <0.001|
| M82362       | C/EBP-α                                                            | 1.3   | 0.01  | 1.52  | <0.001|
| Ribosomal proteins |                                                            |       |       |       |       |
| AU020239     | Ribosomal protein L12                                               | 1.36  | <0.001 | 1.36 | 0.007 |
| M11408       | Ribosomal protein S16                                               | 0.96  | 0.57  | 1.23  | <0.001|
| M14689       | Ribosomal protein L7α                                               | 1.13  | 0.003 | 1.21  | 0.01  |
| Signaling    |                                                                      |       |       |       |       |
| AB020886     | MAP3K4                                                              | 0.96  | 0.8   | 1.22  | <0.001|
| D090163      | Matrix γ-carboxyglutamate (gla) protein                             | 1.48  | 0.06  | 2.59  | <0.001|
| L02914       | Aquaporin 1                                                         | 1.08  | 0.7   | 1.71  | <0.001|
| U35801       | Guanine nucleotide binding protein, α-inhibiting                    | 0.78  | 0.41  | 0.47  | <0.001|
| X00304       | Protein kinase C9                                                   | 1.16  | 0.27  | 1.39  | <0.001|
| Growth factors |                                                            |       |       |       |       |
| L07264       | Heparin binding EGF-like growth factor                              | 1.11  | 0.6   | 1.73  | <0.001|
| M57683       | PDGF receptor                                                      | 1.56  | 0.088 | 2.25  | <0.001|
| Others       |                                                                      |       |       |       |       |
| AF030100     | TNF-related weak inducer of apoptosis                               | 1.15  | 0.26  | 1.81  | <0.001|
| A183217      | Cadherin 5                                                         | 1.72  | 0.03  | 2.06  | <0.001|
| M72333       | Selectin                                                            | 1.39  | 0.3   | 2.74  | <0.001|
| U398261      | Superoxide dismutase 3 (SOD 3)                                      | 0.83  | 0.41  | 0.78  | <0.001|
| X70853       | Fibulin 1                                                           | 1.25  | 0.14  | 1.41  | <0.001|

of the Diabetes Genome Anatomy Project.2 Interestingly, many of the gene expression changes were relatively modest (25–50% increase or decrease); but based on the stringent statistical analysis used for defining these differences, the well defined groups of only one cell type (isolated adipocytes) and the confirmation of at least part of the gene expression changes by real time PCR and proteomic data (10), these are relevant changes in this model.
molecules constituted the largest functional group (28%), followed by genes involved in signaling (18%), transport and trafficking (13%), and substrate and energy metabolism (6%). Except for five genes, a parallel up- or down-regulation of gene expression was observed, which was independent of adipocyte size (Table I).

Most of the genes involved in immune processes, including interferon (IFN)-γ, IFN receptor 2, small inducible cytokine A12 and A4, and P lysozyme were coordinated down-regulated in small and large FIKRO adipocytes in response to the insulin receptor knock-out. Moreover, impaired insulin signaling in adipocytes of FIKRO mice caused a coordinated down-regulation of several genes that play a role in signal transduction, including MAP kinase kinase 1, protein kinase C, the potassium channel KIR 4.2, aquaporin 1, and a voltage-dependent calcium channel (Table I).

In addition, for several significantly changed genes, our data suggest a previously unrecognized regulation by the presence or absence of insulin signaling with three different patterns regulated in FIKRO adipocytes (Fig. 3, A–C). Pattern A (Fig. 3A) is characterized by decreased gene expression in both large and small FIKRO adipocytes compared with controls. This pattern was observed for 17 genes, including MAP kinase kinase 1, protein kinase C, mitochondrial ATP synthase, and TNF-α. Pattern B (Fig. 3B) shows increased gene expression in both large and small FIKRO adipocytes compared with controls and was detected in 17 genes, including aldehyde dehydrogenase 2, insulin-like growth factor I receptor, TGF-β, and 17β-hydroxysteroid dehydrogenase 4. The third pattern, pattern C (Fig. 3C), demonstrated increased gene expression in either small or large control adipocytes with unchanged gene expression in small and large FIKRO adipocytes and was observed for 9 genes, including complement 1q, P lysozyme, CD93 antigen.

**Gene Expression Profiles in Function of the Adipocyte Size**—Comparisons between small and large adipocytes revealed a total of 48 significant differences in cells from FIKRO mice (n = 4) or IR lox/lox mice (n = 45) (Table II). The largest number of these changes were observed for genes in immune response/secreted molecules category (28%), followed by genes involved in transport and trafficking (16%), genes of the substrate and energy metabolism (16%), and transcription factors (12%) (Table II). Of the 48 genes, 38 were up-regulated, and 10 down-regulated in large adipocytes of FIKRO mice (n = 2), IR lox/lox mice (n = 41), or both (n = 6) (Table II). Independent of the genotype, parallel up- or down-regulation was detected for 33 genes, further suggesting an intrinsic heterogeneity in adipocyte gene expression in the function of the adipocyte size.

**Systematic analysis of the gene expression profiles revealed seven distinct gene expression patterns related to cell size (Fig. 3, D–J).** Pattern D was observed for 14 genes and could be characterized by a significant up-regulated gene expression only in small IR lox/lox adipocytes without differences in the other groups (Fig. 3D). This pattern was observed for superoxide dismutase 3 and carbonyl reductase 1. A gene expression pattern with decreased expression in control large adipocytes only (pattern E, Fig. 3E) was observed for 5 genes, including angiotensinogen and ring finger protein 4. Pattern F, with up-regulation in large IR lox/lox adipocytes and indistinguishable gene expression in all other groups (pattern F in Fig. 3F), was the most frequent gene expression pattern and consists of 21 genes, including IL-1β, aquaporin 1, IFN-activated gene 204, which only showed an up-regulation in large IR lox/lox adipocytes, and indistinguishable gene expression in all other groups. Pattern G was observed for 4 genes, including IFN receptor 2, with significantly increased gene expression in small adipocytes of control mice compared with significantly decreased expression in small adipocytes of FIKRO mice (Fig. 3G). Eight genes, including colony-stimulating factor 3 and claudin 5 exhibited pattern H with significantly decreased gene expression only in small adipocytes of IR lox/lox mice compared with the other groups (Fig. 3H). The last two patterns are characterized by a coordinated up- (pattern I, Fig. 3I) or down-regulation (pattern J, Fig. 3J) in large adipocytes independently from the genotype. Pattern I was detected for 13 genes, including lumican, PDGF receptor, cadherin 5, C/EBP-δ, and C/EBP-α, whereas pattern J was observed for pyruvate dehydrogenase. These last two patterns also demonstrate that key adipocyte differentiation markers, i.e. C/EBP-α, C/EBP-δ, as well as genes of the glucose metabolism, i.e. pyruvate dehydrogenase are commonly regulated in function of the adipocyte size, supporting our hypothesis that the differences in triglyceride storage and triglyceride and fatty acid synthesis, as well as differences in insulin sensitivity, between large and small adipocytes might represent intrinsic differences between adipocytes in function of the adipocyte size.

**Consequences of the Impaired Insulin Signaling in FIKRO Adipocytes on the Gene Expression of TNF-α, TGF-β, and PAI-1**—FIKRO mice represent a unique model for differentiating the effects of insulin resistance in adipose tissue from obesity-related insulin resistance in WAT as commonly observed in the metabolic syndrome. These genes would be defined as those altered in the absence of insulin signaling independent of cell size. Our stringent statistical filters revealed that among those genes listed in Table I, ACRP30, TNF-α, TGF-β, and PAI-1 gene expression were significantly associated with the impaired insulin signaling that was independent of cell size (Fig. 4), suggesting a direct relationship between expression of these secreted molecules and insulin resistance in WAT. In each case, the gene expression changes were confirmed by real time PCR. Surprisingly, we observed significantly decreased TNF-α gene expression in isolated adipocytes from FIKRO mice, whereas ACRP30, TGF-β, and PAI-1 gene expression were coordinately increased in both small and large adipocytes from FIKRO mice (Fig. 4). To exclude an effect of collagenase digestion to alter TNF-α gene expression, we confirmed decreased TNF-α gene expression in RNA extracted from undigested adipose tissue from FIKRO mice.

**Consequences of Impaired Insulin Signaling and Adipocyte Size on Gene Expression of Key Adipocyte Candidate Genes**—In an additional analysis of key adipocyte genes, we identified several key categories of adipocyte genes that were regulated as a function of adipocyte size and/or in response to the IR knock-out (Fig. 5). We selected genes for secreted molecules (Fig. 5A), transcription factors that play a role in adipogenesis (Fig. 5B), and genes for the intracellular insulin signaling (Fig. 5C). These categories were identified in the microarray data, and real time PCR was performed to confirm the chip results. In the first group of genes for secreted molecules, we observed expression patterns indicating regulation by the absence or presence of the insulin receptor signaling, but not by the adipocyte size. Thus, leptin and TNF-α gene expression were down-regulated in FIKRO adipocytes of both size groups compared with small and large adipocytes from IR lox/lox mice (Fig. 5A), whereas ACRP30 gene expression was higher in FIKRO adipocytes compared with the controls independently of cell size (Fig. 5A). This result is in accordance with the previously reported increased serum ACRP30 concentrations and the increased ACRP30 protein levels in isolated adipocytes (2).

The analysis of several transcription factors involved in control of the adipogenic program revealed no difference in gene expression for peroxisome proliferator-activated receptor-γ by
Fig. 3. Heterogeneity of gene expression patterns depending on the impaired insulin signaling and the adipocyte size. Differential gene expression in isolated adipocytes depending on cell diameter from 3-month-old male IR (lox/lox) and FIRKO mice. Adipocytes from epididymal fat pads of 8–10 IR lox/lox and 9–13 FIRKO mice were isolated by collagenase I digestion, pooled, and separated into two different subsets of small (IR loxS, FIRKOS) and large (IR loxL, FIRKOL) adipocytes using a nylon mesh of 75-μm pore size. 10 patterns of different protein expression in small and large adipocytes from FIRKO and IR lox/lox mice were observed: decreased gene expression in both large and small FIRKO adipocytes compared with controls (A); increased gene expression in both large and small FIRKO adipocytes compared with controls (B); increased gene expression in both small and large IR lox/lox adipocytes compared with both groups of FIRKO adipocytes with differential levels in large and small IR lox/lox adipocytes (C); up-regulated gene expression only in small IR lox/lox adipocytes without differences in the other groups (D); down-regulated gene expression only in small IR lox/lox adipocytes without differences in the other groups (E); up-regulation only in large IR lox/lox adipocytes, with indistinguishable gene expression in all other groups (F); increased gene expression in small IR lox/lox adipocytes compared with all other groups and an additional significantly down-regulated gene expression in small FIRKO adipocytes compared with all other groups (G); decreased gene expression only in small IR lox/lox adipocytes compared with the other groups (H); coordinated up-regulation in large adipocytes independently from the genotype (I); coordinated down-regulation in large adipocytes independently from the genotype (J). For each of the 10 patterns representative microarray data ± S.D. from at least five independent experiments are shown. *, p < 0.05 in FIRKO versus IR lox; #, p < 0.05 in large versus small adipocytes.
cell size or insulin signaling (Fig. 5B). However, C/EBP-β gene expression was regulated as a function of the adipocyte size, i.e. decreased both in small adipocytes from FIRKO and IR lox/lox mice (Fig. 5B). C/EBP-β expression, on the other hand, was only down-regulated in small FIRKO adipocytes (Fig. 5B). In the group of genes involved in insulin signaling, only IRS-1 was significantly higher expressed in small adipocytes of IR lox/lox mice compared with other groups (Fig. 5C). There was no difference in the gene expression of IRS-2 or phosphatidylinositol 3-kinase between the groups (Fig. 5C). In a parallel study using proteomics (10), we found alteration of a fourth group of genes, key enzymes of lipid metabolism, including fatty acid synthase and carnitinepalmitoyl transferase-2, which had significantly lower protein levels in small adipocytes of both FIRKO and IR lox/lox mice. These changes were accompanied by lower gene expression in small adipocytes of both FIRKO and IR lox/lox mice (p < 0.01) but did not meet the criteria of the third statistical filter (p < 0.0001) and therefore do not appear in the list of significantly changed genes.

DISCUSSION

Although adipose tissue is only a minor site of glucose uptake, adipose tissue plays an important role in overall glucose homeostasis. Insulin resistance is associated with both obesity (17) and various syndromes of lipodystrophy (18) and is also observed in mice with a fat-specific knock-out of GLUT4 (19). By contrast, we have shown that adipose tissue-specific disruption of the insulin receptor in FIRKO mice has beneficial effects on both whole body glucose homeostasis and longevity (2, 3). FIRKO mice have reduced total body fat mass and are protected against age-related obesity and its related metabolic abnormalities (2). Adipose tissue of FIRKO mice displays a heterogeneity of adipocyte size with polarization into small and large adipocytes. This is accompanied by changes in lipid storage function, glucose metabolism, and the expression of major modulators of adipocyte metabolism (fatty acid synthase), secretory function (ACRP30), and adipocyte differentiation (SREBP-1, C/EBP-α) (2). Moreover, in parallel to the detected differences in adipocytes of FIRKO mice, we observed differences with respect to glucose transport, lipid storage function, and glucose metabolism in small and large adipocytes of control mice (2). We therefore hypothesized that the impaired insulin signaling in FIRKO adipocytes unmasks an intrinsic heterogeneity in WAT, which might account for the observed differences in physiologic parameters. Our hypothesis is further supported by the development of similar heterogeneity of WAT in mice lacking the enzyme hormone-sensitive lipase (4, 5), mice with a genetic knock-out of perilipin (20), and by the differences in biology between adipocytes in different white fat depots, such as visceral and subcutaneous fat (21).

In the present study, we have begun to define the alterations in gene expression that might contribute to the physiologic differences in adipocyte physiology in function of the cell size. WAT of FIRKO mice provides a unique model in which to investigate the consequences of impaired insulin signaling and the role of adipocyte size on changes in gene and protein expression patterns, which might be involved in regulation of adipose biology in vivo and lead to heterogeneity of adipocytes in FIRKO mice.
As a first approach, we screened mRNA expression using oligonucleotide microarrays for important adipocyte genes and genes that are involved in insulin signaling. We found that impaired insulin signaling results in decreased leptin and TNF-α mRNA expression, whereas ACRP30 gene expression is up-regulated in accordance with previous data on protein and serum concentrations (2). Gene expression analysis also confirmed the previous finding that despite the disruption of the insulin receptor, the expression of insulin signaling molecules, e.g. IRS-1, IRS-2, phosphatidylinositol 3-kinase, and others is not changed, whereas other signaling molecules, i.e. MAP kinase 1 or protein kinase Cβ are regulated differentially because of the IR knock-out. Moreover, several transcription factors that have important functions in adipogenesis, i.e. C/EBP-α, C/EBP-β, and SREBP-1c, are regulated differentially in small and large adipocytes either in one genotype or in both. Interestingly, insulin-like growth factor I receptor gene expression was significantly up-regulated in FIRKO adipocytes, although, insulin-like growth factor I receptor expression at the protein level was indistinguishable between the genotypes (2).

In this study, we demonstrated that differential regulation in gene expression in control and FIRKO adipocytes could be related to both the cell size and impaired insulin signaling. More importantly our analysis not only allowed us to screen the microarray data for candidate genes, but also to define genes with a previously unrecognized connection to insulin signaling and adipocyte biology. We propose that functional differences, specific gene expression patterns, and specific changes in protein levels, as determined by a proteomics approach (10), might define different subclasses of adipocytes, not only in adipose tissue of FIRKO mice, but also in fat of normal mice, where 92% of the significant changes in gene expression between large and small adipocytes were also found. A complete list of alterations at the mRNA levels as a function of the cell size and the impaired insulin signaling can be obtained from the Diabetes Genome Anatomy Project Web site.

Of the changes in gene expression, 48 were coordinately up- or down-regulated because of the disruption of insulin signaling. Most of the gene expression changes occurred in genes involved in immune response processes. Thus, we found a down-regulation of the TNF-α, IFN-γ, small inducible cytokines A12 and A4, and complement 1q component gene expression in FIRKO adipocytes. The down-regulation of cytokine

![Image](https://example.com/image.png)
gene expression in adipocytes of FIRKO mice might be the result of the lean phenotype of these mice, because in the opposite situation, i.e. obesity, an up-regulation of TNF-α (22), IFN-γ (23) and other cytokines has been reported. Interestingly, mice lacking the TNF-α function are protected against obesity-related insulin resistance (24), suggesting that decreased TNF-α gene expression could contribute to the protection against obesity in FIRKO mice. These changes were not simply the result of the collagenase digestion process used to isolate adipocytes because decreased TNF-α mRNA could also be found in undigested adipose tissue of FIRKO versus control mice.

FIRKO mice also provide a model for investigation of the regulation of TNF-α, TGF-β, and PAI-1 in adipose tissue-specific insulin resistance. TNF-α and TGF-β are potent inducers of PAI-1 expression, and in severe obesity a proportional increase in adipose tissue PAI-1 and TGF-β has been reported in humans and mice (25, 26). It has been further suggested that increased TNF-α provides the common link between insulin resistance and elevated PAI-1 and TGF-β expression in obesity (26). However, in adipocytes from FIRKO mice, PAI-1 and TGF-β gene expression were found to be up-regulated despite significantly lower TNF-α expression, suggesting that impaired insulin signaling can affect PAI-1 and TGF-β gene expression independently of increased TNF-α expression.

Another group of differentially regulated genes are molecules of the intracellular signaling system. We found a down-regulated protein kinase Cδ and MAP kinase kinase 1 gene expression in FIRKO adipocytes, independent of the cell size. Protein kinase Cδ has been shown to mediate insulin-induced glucose transport in primary cultures of rat skeletal muscle, thus representing a major link between insulin signaling and glucose transport (27).

Analysis of gene expression patterns as a function of the adipocyte size revealed profound differences between small and large adipocytes. Although small adipocytes might simply represent the natural precursors of large adipocytes, our data suggest that this is not strictly the case. This includes the relatively uniform gene expression of leptin and ACRP30 as well as typical gene expression of terminally differentiated adipocytes, i.e. no changes in peroxisome proliferator-activated receptor-γ, GLUT4, and aP2 gene expression both in large and small adipocytes of FIRKO mice, suggesting that heterogeneity in adipose tissue of FIRKO mice is not the result of different stages of adipocyte differentiation.

The differences in the gene expression patterns of secreted molecules, including angiotensinogen, IL-1β, granulocyte colony-stimulating factor 3, and complement 1 may be important for different endocrine function in adipocytes of different size. IL-1β is a potent inhibitor of adipogenesis (28) and is up-regulated in small adipocytes independently from the genotype. Thus, increased IL-1β secretion from small adipocytes might contribute to the regulation of adipogenesis and secondary regulation of adipose tissue mass. In addition, IL-1β stimulates the 11β-hydroxysteroid dehydrogenase (29). Overexpression of 11β-hydroxysteroid dehydrogenase has been shown to cause visceral obesity and a metabolic syndrome in mice (30).

Of those genes that were significantly down-regulated in small adipocytes of both FIRKO and control mice, angiotensinogen is of interest because angiotensin type 1 receptors play a role in the in vitro differentiation of human preadipocytes. Decreased angiotensinogen expression in small adipocytes could thereby contribute to a potential feedback mechanism in the stimulation or inhibition of adipogenesis.

We further demonstrate coordinated up-regulation of PDGF receptor mRNA in small adipocytes of both genotypes. In cultured adipocytes PDGF is one of the most potent stimulators of proliferation but requires the activation-dependent expression of its receptor protein (31). PDGF receptor down-regulation has been shown to be an important marker for terminally differentiated adipocytes (32), suggesting that small adipocytes might either have an increased intrinsic differentiation capacity or represent indeed premature adipocytes.

In addition to the differentially secreted and signaling molecules, we identified differentially expressed genes that encode proteins involved in the immune response, cytoskeleton, metabolism, and apoptosis which might define the small and large adipocyte phenotypes. Surprisingly, several genes of the energy and substrate metabolism, including fructose-6-phosphatase, lactate dehydrogenase 1, and protein glycosyltransferase, are down-regulated in large adipocytes, whereas others, i.e. pyruvate decarboxylase and carnobyl reductase 1, are significantly up-regulated in large adipocytes, suggesting that small and large adipocytes represent intrinsic adipocyte subclasses.

Finally six transcription factors, including C/EBP-α and C/EBP-δ were identified among the significantly differently expressed genes in small and large adipocytes. The role of C/EBP-α and C/EBP-δ in adipogenesis is well characterized; either is sufficient to mediate the adipogenic program (7, 33). The coordinated down-regulation of these transcription factors in small adipocytes of both genotypes while other markers of terminally expressed adipocytes are normally expressed is also consistent with an intrinsic heterogeneity in the adipocyte differentiation program.

In conclusion, knock-out of the insulin receptor in fat unmask an intrinsic heterogeneity in adipocyte size and physiologic function accompanied by changes in patterns of gene expression that may be involved in the regulation of adipocyte differentiation, triglyceride storage, and adipocyte metabolism. Using a genomics approach, we identified classes of genes that are regulated either by the adipocyte size, by the impaired insulin signaling, or by both. These changes in gene expression may define these unique populations of adipocytes and potential novel therapeutic targets for the treatment or prevention of obesity.

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