Gene expression and biological processes influenced by deletion of Stat3 in pulmonary type II epithelial cells

Yan Xu*, Machiko Ikegami, Yanhua Wang, Yohei Matsuzaki and Jeffrey A Whitsett

Address: Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, OH, USA

Email: Yan Xu* - yan.xu@cchmc.org; Machiko Ikegami - machiko.ikegami@cchmc.org; Yanhua Wang - yanhua.wang@cchmc.org; Yohei Matsuzaki - yohei.matsuzaki@cchmc.org; Jeffrey A Whitsett - jeff.whitsett@cchmc.org

* Corresponding author

Abstract

Background: The signal transducer and activator of transcription 3 (STAT3) mediates gene expression in response to numerous growth factors and cytokines, playing an important role in many cellular processes. To better understand the molecular mechanisms by which Stat3 influences gene expression in the lung, the effect of pulmonary epithelial cell specific deletion of Stat3 on genome wide mRNA expression profiling was assessed. Differentially expressed genes were identified from Affymetrix Murine GeneChips analysis and subjected to gene ontology classification, promoter analysis, pathway mapping and literature mining.

Results: Total of 791 mRNAs were significantly increased and 314 mRNAs were decreased in response to the deletion of Stat3Δ/Δ in the lung. STAT is the most enriched cis-elements in the promoter regions of those differentially expressed genes. Deletion of Stat3 induced genes influencing protein metabolism, transport, chemotaxis and apoptosis and decreased the expression of genes mediating lipid synthesis and metabolism. Expression of Srebf1 and 2, genes encoding key regulators of fatty acid and steroid biosynthesis, was decreased in type II cells from the Stat3Δ/Δ mice, consistent with the observation that lung surfactant phospholipids content was decreased. Stat3 influenced both pro- and anti-apoptotic pathways that determine cell death or survival. Akt, a potential transcriptional target of Stat3, was identified as an important participant in Stat3 mediated pathways including Jak-Stat signaling, apoptosis, Mapk signaling, cholesterol and fatty acid biosynthesis.

Conclusion: Deletion of Stat3 from type II epithelial cells altered the expression of genes regulating diverse cellular processes, including cell growth, apoptosis and lipid metabolism. Pathway analysis indicates that STAT3 regulates cellular homeostasis through a complex regulatory network that likely enhances alveolar epithelial cell survival and surfactant/lipid synthesis, necessary for the protection of the lung during injury.
Background
The signal transducers and activators of transcription (STATs) were initially identified as a family of latent cytoplasmic transcription factors that are activated by various cytokines, growth factors and other stimuli, and phosphorylated by many protein kinases [1-4]. In response to various stimuli, STAT family members are phosphorylated by receptor associated kinases, form homo- or heterodimers and are translocated to the cell nucleus where they activate transcription [5]. Recent studies also support the role of unphosphorylated STAT3 that accumulates in nucleus and activates transcription by binding to NFκB [6]. STAT3 regulates a variety of biological processes, functioning at both transcriptional and non-transcriptional levels to influence cell growth, survival and metabolism. Its capacity to induce cell transformation and tumorigenesis makes it a potential therapeutic target for various cancers [7,8].

Systemic deletion of Stat3 is embryonic lethal in the mouse, indicating its important role in embryogenesis [9]. Biological roles of STAT3 in various organs and cells have been studied in vitro as well as cell specific deletion in the mouse in vivo. The biological consequences of Stat3 deletion are surprisingly diverse and sometimes contradictory. For example, cardiomyocyte-specific STAT3 deficiency caused cardiac fibrosis and heart dysfunction with advanced age [10]. Hepatic cell specific deletion of Stat3 caused insulin resistance associated with increased expression of gluconeogenic genes [11]. Conditional ablation of Stat3 in respiratory epithelium of the mouse (Stat3Δ/Δ mice) did not alter lung morphogenesis or function but enhanced susceptibility to hyperoxia and adenoviral induced lung injury whereas overexpression of Stat3C (a constitutive active form of STAT3) in pulmonary epithelium protects against hyperoxic lung injury, suggesting that STAT3 is required for the maintenance of surfactant homeostasis and lung function during injury [12-14]. While STAT3 has been proposed as an anti-apoptotic protein through the induction of survival genes such as Bcl2-like 1 (Bcl-x) and B-cell leukemia/lymphoma 2 (Bcl-2), STAT3 also exerts pro-apoptotic effect through the regulation of insulin-like growth factor binding protein 5 (IGFBP-5) to modulate mammary epithelial apoptosis [4,15]. STAT3 is abundantly and ubiquitously expressed in many tissues and distributed between the cell cytosol and nucleus. A direct effect of non-phosphorylated, cytoplasmic STAT3 on cell motility was reported recently through direct protein-protein interactions [16], indicating a non-transcriptional function of STAT3.

The functions of STAT3 vary in different cellular and physiological contexts, influencing diverse gene targets by interaction with other proteins and genes. The diversity of STAT3 functions indicate that STAT3 is involved in complex genetic networks to maintain cellular homeostasis rather serving a singular role in acute phase responses as initially defined. In the present study, we sought to systematically study the role of STAT3 in pulmonary epithelial cell homeostasis. Using knowledge based gene expression profiling approaches and a conditional system that selectively deleted Stat3 in the respiratory epithelium; we identified a large STAT3-dependent network that influences a wide variety of biological processes in type II alveolar cells in the lung.

Results and Discussion
Identification of differentially expressed genes in alveolar type II epithelial cells from Stat3Δ/Δ mice
In previous studies Stat3 mRNA and protein expression were markedly reduced in Type II cells isolated from Stat3Δ/Δ mice, being less than 10% of control levels [12]. To identify the RNAs influenced by the conditional deletion of Stat3Δ/Δ, RNAs isolated from alveolar epithelial type II cells of control and Stat3Δ/Δ mice were compared using Affymetrix murine genome MOE430 gene chips. The complete dataset can be found at Gene Expression Omnibus (GEO) [17]; Accession no. GSE6846. Total of 1105 genes were identified as significantly altered using the criteria described in Method. Among them, 791 mRNAs were increased and 314 mRNAs were decreased in response to the deletion of Stat3Δ/Δ in the lung (Additional file 1). Changes in mRNA expression of a subset of genes including Malt1, Rnt4, Reg3g, Bcl2l1, Cds2, Cdk7, Fasn, Acox2, Akt2, Gpam, Foxj1, Abca3, Srebfl, Sreb2 and Scap were validated by real-time RT-PCR. Genes cross validated by RT-PCR are listed in Table 1 and indicated by asterisks (*) in Additional file 1.

Functional classification of differentially expressed genes revealed the dysregulation of various biological processes in type II epithelial cells from Stat3Δ/Δ mice
Differentially expressed genes were classified according to Gene Ontology (GO) classification on Biological Process. The Fisher Exact Test was used to calculate the probability of each category that was overrepresented in the selected list using the entire MOE430 mouse genome as reference dataset. Deletion of Stat3 from type II cells significantly induced the genes involved in protein metabolism, protein transport, chemotaxis and apoptosis and decreased the expression of genes in lipid synthesis and metabolism (Table 2).

Promoter analysis identified putative common regulators of the differentially expressed genes
To identify putative common transcription factors regulating the type II cell responses to Stat3 deletion, promoter region (-2kb to exon1) of differentially expressed genes were searched for overrepresented cis-elements using MatInspector (Genomatix) vertebrate matrix library. In com-
pare with the sequence of random chosen gene promoters, the cis-elements significantly enriched in the promoter region of differentially expressed genes were selected based on a binomial probability calculation and their percentage frequency in our selected gene list (Adjusted p Value < 0.001 and frequency > 50 %). STAT, EGRF, AHRR, SP1F, ZF5F, E2FF, H1FF, SREB and AP2F were the most overrepresented cis-elements and may therefore mediate changes in gene expression in cells from Stat3Δ/Δ mice (Table 3). The finding that STAT was the

Table 1: Comparison of mRNAs by RT-PCR and RNA microarray

| GENE      | RT-PCR | Microarray | P-Value for RT-PCR | Gene Name                                                                 |
|-----------|--------|------------|--------------------|---------------------------------------------------------------------------|
| Malt1     | 3.5    | 6.27       | < 0.050            | Mucosa associated lymphoid tissue lymphoma translocation gene 1            |
| Rtn4      | 2.7    | 4.86       | < 0.050            | recitulin 4                                                               |
| Reg3g     | -5.5   | -6.02      | < 0.050            | regenerating islet-derived 3 gamma                                        |
| Bcl2I     | -1.2   | -1.54      | 0.053              | Bcl2-like 1                                                               |
| AxC2      | -1.5   | -1.58      | < 0.050            | thymoma viral proto-oncogene 2                                            |
| ABOcA3    | -2.4   | -1.59      | < 0.050            | ATP-binding cassette, sub-family A (ABC1), member 3                        |
| Scap      | -1.4   | -1.58      | < 0.050            | SREBP cleavage activating protein                                         |
| Srebf1    | -1.4   | -1.69      | < 0.050            | sterol regulatory element binding factor 1                                |
| Srebf2    | -1.3   | -1.50      | 0.080              | sterol regulatory element binding factor 2                                 |
| Cdipt     | -1.8   | -1.54      | < 0.001            | CDP-diacylglycerol--inositol 3-phosphatidyltransferase                     |
| Fasn      | -1.7   | -1.57      | < 0.001            | fatty acid synthase                                                       |
| Acox2     | -1.5   | -1.67      | < 0.001            | acyl-Coenzyme A oxidase 2                                                 |
| Cds2      | -2.1   | -1.78      | < 0.001            | CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase)          |
| Gpam      | -1.5   | -1.54      | < 0.001            | glycerol-3-phosphate acyltransferase                                      |

Note: Fold change of mRNA in Stat3Δ/Δ mice to control is shown.

Table 2: Functional Classification of Genes Differentially Expressed in Stat3Δ/Δ mice

| GO Classification of Genes Up-regulated in Stat3Δ/Δ mice |
|---------------------------------------------------------|
| Term                             | Count | %    | PValue         |
| Biopolymer modification          | 115   | 13.94| 7.30E-13       |
| Cellular protein metabolism      | 164   | 19.88| 5.83E-10       |
| Protein transport                | 52    | 6.30 | 2.49E-07       |
| Phosphate metabolism             | 62    | 7.52 | 3.31E-07       |
| Chemotaxis                      | 17    | 2.06 | 1.38E-06       |
| Protein kinase cascade           | 24    | 2.91 | 6.83E-06       |
| Apoptosis                       | 40    | 4.85 | 1.69E-05       |
| Regulation of apoptosis          | 29    | 3.52 | 4.93E-05       |
| Positive regulation of cellular metabolism | 25   | 3.03 | 9.95E-05       |
| Cell migration                   | 19    | 2.30 | 2.26E-03       |
| Transcription                    | 106   | 12.85| 2.64E-03       |

| GO Classification of Genes Down-regulated in Stat3Δ/Δ mice |
|----------------------------------------------------------|
| Term                             | Count | %    | PValue         |
| Cellular lipid metabolism        | 33    | 9.97 | 3.60E-13       |
| Lipid biosynthesis               | 21    | 6.34 | 3.59E-11       |
| Sterol metabolism                | 10    | 3.02 | 2.96E-07       |
| Fatty acid metabolism            | 14    | 4.23 | 5.35E-07       |
| Steroid metabolism               | 12    | 3.63 | 6.83E-06       |
| Carboxylic acid metabolism       | 18    | 5.44 | 5.84E-04       |
| Coenzyme metabolism              | 10    | 3.02 | 2.51E-03       |
| Cellular carbohydrate metabolism | 12    | 3.63 | 3.98E-03       |
| Electron transport               | 14    | 4.23 | 2.64E-02       |
| Phosphate metabolism             | 21    | 6.34 | 3.27E-02       |

Note: Gene Ontology Analysis was preformed using public available web-based tool DAVID [81]. Overrepresented biological processes were selected at the threshold of Fisher Exact Test P-Value ≤ 0.05 and minimum gene counts belonging to an annotation term ≥ 2%.
Analysis of differentially expressed genes using the Pathway Enrichment test revealed known and novel functions of Stat3 in lung epithelial cells. Sterol regulatory element binding factors (Srebf1 and 2), known as important transcriptional regulators of fatty acid and steroid biosynthesis, were significantly decreased in Stat3Δ/Δ mice. The SREB binding site was overrepresented in differentially expressed genes indicating that it is a potential regulator of the lipid metabolism pathways altered in Stat3Δ/Δ mice. Other significantly enriched TFBS include SP1F and HIFF. Stat3 and SP1 function cooperatively to activate the C/EBP promoter, the SP1 site being required for IL-6 induction and transactivation by STAT3 [18]. HIF1A, SP1, SMAD3 AND SMAD4 can form multifactor complex, regulating VEGF and erythropoietin gene transcription through functional cooperation and association [19-21]. Consistent with the promoter analyses and literature findings, the mRNA expression of Klf5 (a member of the SP1 family) and Hif1a, Smad3 and Smad4 was simultaneously increased by Stat3 deletion, indicating the potential transcriptional complex formation among the corresponding transcription factors.

Pathway analysis revealed known and novel functions of STAT3 in the lung

Pathway enrichment test is an unbiased way to answer the question, “Are the differentially expressed genes enriched in certain pathways?” To address this issue, we compared the overlap of differentially expressed genes in Stat3Δ/Δ mice with the known biological pathways in KEGG (Kyoto Encyclopedia of Genes and Genomes) using 1) the mouse genome and 2) a list of genes shown to be least changed in response to the Stat3 deletion in the gene array as background. Results from both analyses were consistent. Jak-Stat Signaling Pathway, Apoptosis, Cytokine-Cytokine Receptor Interaction, Insulin Signaling Pathway, Mapk Signaling Pathway, Focal Adhesion, and Wnt Signaling Pathway were among the most enriched pathways identified from the analysis of differentially expressed genes (Table 4). mRNAs mediating steroid biosynthesis and fatty acid metabolism were mostly decreased in Stat3Δ/Δ mice. The most overrepresented pathways and the differentially expressed genes associated with those pathways are illustrated in Additional files 2. The finding that known Stat3 functions, including the Jak-Stat Signaling Pathway were identified from microarray analysis of Stat3Δ/Δ mice type II cells using knowledge integration approaches provides support for the utility of the analysis to detect novel pathways regulated by Stat3.

Stat3 influences protein metabolism in lung type II cells

“Protein metabolism” was the most enriched biological process (5.83E-10), accounting for 20% of the induced genes caused by the deletion of Stat3. More than 40 genes encoding proteins involved in protein ubiquitination/ubiquitin cycle were present (Table 5). Of interest, Casitas B-lineage lymphoma b (Cblb) was increased 7.3 fold. CBLB is a member of Cbl ubiquitin ligases (E3) protein family that are tyrosine-phosphorylated in response to a

| Term                          | Count | %     | P-Value |
|-------------------------------|-------|-------|---------|
| Jak-Stat Signaling Pathway     | 38    | 3.26  | 7.56E-09|
| Apoptosis                     | 24    | 2.06  | 5.04E-06|
| Cytokine-Cytokine Receptor Interaction | 43 | 3.69  | 2.59E-05|
| Insulin Signaling Pathway     | 28    | 2.40  | 5.53E-05|
| Mapk Signaling Pathway        | 45    | 3.86  | 8.51E-05|
| Focal Adhesion                | 37    | 3.17  | 1.35E-04|
| Wnt Signaling Pathway         | 28    | 2.40  | 1.68E-04|

Overrepresented pathways were identified by comparison the overlap of differentially expressed genes and all genes in MOE430 mouse genome (reference) with the known KEGG pathways. A pathway is considered to be over-represented when a probability P-Value ≤ 0.01 and gene frequency (genes in the pathway/total number of differentially expressed genes) ≥ 2%.

Table 3: Enriched TFBS In Genes Differentially Expressed in Stat3Δ/Δ mice

| TFBS   | Count (Diff_Gene) | Frequency (Diff_Gene) | Count (background) | Frequency (background) | P-Value | Q-Value |
|--------|-------------------|-----------------------|--------------------|------------------------|---------|---------|
| V$STAT| 1056              | 0.97                  | 786                | 0.79                   | 4.93E-59| 2.29E-56|
| V$EGFRF| 1056             | 0.96                  | 757                | 0.76                   | 2.92E-57| 1.35E-54|
| V$AHRR| 1056              | 0.90                  | 544                | 0.54                   | 4.04E-53| 1.87E-50|
| V$SP1F| 1056              | 0.92                  | 789                | 0.79                   | 2.15E-49| 9.96E-47|
| V$ZF5F| 1056              | 0.97                  | 321                | 0.32                   | 2.18E-48| 1.01E-45|
| V$SE2FF| 1056             | 0.96                  | 778                | 0.78                   | 1.30E-47| 6.03E-45|
| V$HIFF| 1056              | 0.99                  | 439                | 0.44                   | 4.66E-30| 2.16E-27|
| V$SREB| 1056              | 0.97                  | 425                | 0.42                   | 2.76E-17| 1.28E-14|
| V$AP2F| 1056              | 0.96                  | 420                | 0.42                   | 4.93E-15| 2.29E-12|

Note: promoter sequence (-2000 – 0 bps) from 886 out of 1105 differentially genes were available for analysis. The P-Value is calculated using binomial distribution probability. 5 random sets of 1000 mouse gene -2kb promoter regions were used to calculate the background frequency. The Q-Value is calculated using single-step Bonferroni adjustment to control for the multiple comparison effect.
wide variety of receptor mediated stimuli, including epi-
dermal growth factor receptors, cytokine receptors such as
colony stimulating factor family receptors (increased 2–3
fold in the Stat3Δ/Δ cells) and immune complex receptors
such as Fcgr2b (increased 2.7 fold). CBLB functions as an
adaptor protein that regulates many signal-transduction
pathways including JAK-STAT signaling pathways through
its ability to interact with critical signaling molecules [22].
The expression of genes encoding known CBLB binding
partners was significantly increased in type II cells from
the Stat3Δ/Δ mice, including SYK kinase (increased 4 fold),
JAK2 (increased 2.1 fold), PKC (increased 2.6 fold), phos-
phatidylinositol 3-kinase (Pik3r1 increased 1.8 fold,
Pik3c2a increased 3 fold), adenylate cyclase-associated
protein 1 (Cap1 increased 5 fold) and Crk (increased 2.5
fold). These signaling molecules can in turn modulate var-
ious downstream signal cascades including those
mediated by small G-proteins (multiple members of RAS
oncoprotein family were induced 2–3 fold), as well as PI3K/
AKT mediated signaling and Nfat induction (increased 4.1
fold) [23-27]. CBLB physically interacts with another
ubiquitin-protein ligase, NEDD4 (increased 3.8 fold), an
interaction that reverses CBLB effects by targeting CBLB
for ubiquitination and proteosomal degradation [28].
SLIM is a known E3 ubiquitin ligase specifically interacts
with activated STAT1 and STAT4, inducing their ubiquiti-
nation and degradation [29]. We speculate that CBLB may
act in a similar manner to regulate STAT3 signaling. In
addition, SUMO1 and SUMO1 specific peptidase 2 were
both increased more than 2 fold in Stat3Δ/Δ mice. SUMO1
can covalently modify many targets including STAT1 and
glucocorticoid receptor NR3C1 (increased 5 fold) and reg-
ulate protein stability and transcriptional activity [30].
Taken together, deletion of STAT3 activates a number of
molecules regulating protein metabolism, stability and
routing, indicating the potential role of ubiquitination
and sumoylation in cytokine signaling and STAT3 activa-
tion that influences cellular adaptation.

**STAT3 influences phosphate metabolism and protein
kinase cascade in lung type II cells**

Expression of genes regulating "Phosphate metabolism"
and "protein kinase cascade" were significantly increased in
Stat3Δ/Δ mice (3.1E-07 and 1.38E-06, respectively),
accounting for 7.5% of total induced genes (Table 6). Expression of genes encoding a number of kinase that

### Table 5: Genes In Ubiquitin Cycle Were Largely Induced In Stat3Δ/Δ Mice

| UniGene ID | Fold | P-Value | Gene Symbol | Gene Title |
|------------|------|---------|-------------|------------|
| Mm.328206  | 7.29 | 9.98E-04| Cblb        | Casitas B-lineage lymphoma b |
| Mm.260635  | 4.56 | 6.34E-03| Axot        | Axotrophin |
| Mm.98668   | 3.79 | 6.93E-05| Nedd4l      | neural precursor cell expressed, developmentally down-regulated gene 4-like |
| Mm.276229  | 3.37 | 1.41E-03| Fbxo30      | F-box protein 30 |
| Mm.258476  | 3.30 | 6.58E-03| Tbl1x       | transducin (beta)-like 1 X-linked |
| Mm.28017   | 3.22 | 3.62E-03| Fbxw11      | F-box and WD-40 domain protein 11 |
| Mm.16974   | 3.03 | 1.66E-04| Usp47       | ubiquitin specific peptidase 47 |
| Mm.389965  | 3.01 | 8.38E-04| Rnf12       | Ring finger protein 12 |
| Mm.41711   | 2.87 | 1.43E-03| Pja2        | Prja 2, RING-H2 motif containing (Pja2), transcript variant 2, mRNA |
| Mm.214746  | 2.75 | 1.78E-03| Fbx13       | F-box and leucine-rich repeat protein 3 |
| Mm.242646  | 2.75 | 1.83E-03| Usp9x       | ubiquitin specific pro tease 9, X chromosome |
| Mm.9002    | 2.70 | 6.26E-04| Ube3a       | ubiquitin protein ligase E3A |
| Mm.30051   | 2.53 | 4.86E-04| Rnf10       | Ring finger protein 10 |
| Mm.362118  | 2.41 | 9.26E-04| Sumol       | SMT3 suppressor of mif two 3 homolog 1 (yeast) (Sumo1) |
| Mm.328135  | 2.36 | 3.35E-04| Rfwd2       | Constitutive photomorphogenic protein (Cop1) |
| Mm.290908  | 2.34 | 3.27E-04| Birc6       | baculoviral IAP repeat-containing 6 |
| Mm.180052  | 2.34 | 4.84E-03| Ube2d2      | Ubiquitin-conjugating enzyme E2D 2 |
| Mm.391601  | 2.29 | 9.75E-03| Ube2d3      | ubiquitin-conjugating enzyme E2D 3 |
| Mm.4480    | 2.25 | 1.28E-03| Rbbp6       | Retinoblastoma binding protein 6 (Rbbp6) |
| Mm.319512  | 2.24 | 5.65E-03| Hip2        | huntingtin interacting protein 2 |
| Mm.12665   | 2.19 | 1.60E-02| Cul3        | cullin 3 |
| Mm.244179  | 2.12 | 3.75E-03| Herc1       | Hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1 |
| Mm.392272  | 2.10 | 4.55E-04| Smurf2      | SMAD specific E3 ubiquitin protein ligase 2 |
| Mm.297431  | 1.95 | 2.84E-03| Senp2       | SUMO/sentrin specific peptidase 2 |
| Mm.253542  | 1.94 | 1.53E-02| Rnf138      | ring finger protein 138 |
| Mm.235407  | 1.94 | 1.32E-02| Ube2v2      | ubiquitin-conjugating enzyme E2 variant 2 |
| Mm.392862  | 1.93 | 3.54E-03| Arhi1       | aridene ubiquitin-conjugating enzyme E2 binding protein homolog |
| Mm.78812   | 1.89 | 1.07E-02| Wwp1        | WW domain containing E3 ubiquitin protein ligase 1 |
| Mm.44876   | 1.84 | 3.88E-03| Trim2       | tripartite motif protein 2 |
phosphorylate STAT3 in vivo or in vitro were increased [31-35], including Janus kinase 1 and 2 (increased 3.1 and 2.6 fold, respectively), ribosomal protein S6 kinase polypeptide 3 (Rps6ka3, increased 3.1 fold), met proto-oncogene (Met, increased 4.2 fold), mitogen activated protein kinase 8 (Mapk8, increased 4.7 fold) and Dual-specificity tyrosine-phosphorylation regulated kinase 1a (Dyrk1a, increased 2.6 fold). The increased expression of these genes indicates a potential compensatory mechanism related to the lack of activation of STAT3 or its targets.

**STAT3 influences lipid homeostasis in lung type II cells**
Expression of genes encoding sterol regulatory element binding factor 1 and 2 (Srebf1 and Srebf2), their cleavage activating protein (Scap) and multiple SREBP target genes involved in lipid metabolism were decreased in Stat3Δ/Δ mice type II cells. As depicted in Figure 1, genes dedicated to the biosynthesis of fatty acid, phospholipid and cholesterol metabolism were largely induced in Stat3Δ/Δ mice, including adenosine kinase (Adk), mitogen activated protein kinase 8 (Mapk8), hypoxia inducible factor 1 alpha subunit (Hif1a), met proto-oncogene (Met), calcium/calmodulin-dependent serine protein kinase (Cask), calcium/calmodulin-dependent protein kinase II, delta (Mapk8), and dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a (Dyrk1a). The decreased expression of these genes indicates a potential compensatory mechanism related to the lack of activation of STAT3 or its targets.

**Table 6: Genes In Phosphate Metabolism Were Largely Induced In Stat3Δ/Δ Mice**

| UniGene ID | Fold | P-Value | Gene Symbol | Gene Title |
|------------|------|---------|-------------|------------|
| Mm.188734  | 8.32 | 3.47E-05| Adk         | Adenosine kinase |
| Mm.21495   | 4.70 | 1.82E-03| Mapk8       | Mitogen activated protein kinase 8 |
| Mm.3879    | 4.69 | 4.29E-05| Hif1a       | Hypoxia inducible factor 1, alpha subunit |
| Mm.86844   | 4.18 | 1.89E-03| Met         | Met proto-oncogene |
| Mm.225505  | 4.09 | 2.32E-03| Chka        | Choline kinase alpha |
| Mm.375031  | 4.02 | 1.39E-03| Syk         | Spleen tyrosine kinase |
| Mm.327591  | 3.60 | 1.26E-03| Cask        | Calcium/calmodulin-dependent serine protein kinase |
| Mm.248647  | 3.42 | 2.36E-02| Prkca       | Phosphatidylinositol 4-kinase type 2 beta |
| Mm.255822  | 3.31 | 2.08E-04| Camk2d     | Calcium/calmodulin-dependent protein kinase II, delta |
| Mm.25559   | 3.30 | 7.41E-01| Stk1b       | Serine/threonine kinase 17b (apoptosis-inducing) |
| Mm.328476  | 3.15 | 1.45E-02| Rps6ka3     | Ribosomal protein S6 kinase polypeptide 3 |
| Mm.289657  | 3.14 | 1.66E-04| Jak1        | Janus kinase 1 |
| Mm.3810    | 3.08 | 7.64E-03| Pik3c2a     | Phosphatidylinositol 3-kinase, C2 domain |
| Mm.202606  | 3.04 | 1.02E-04| Mast4       | Microtubule associated serine/threonine kinase family member 4 |
| Mm.309867  | 2.88 | 3.93E-04| Ptk9        | Protein tyrosine kinase 9 |
| Mm.280125  | 2.86 | 3.15E-03| Crk         | v-crk sarcoma virus CT10 oncogene homolog (avian) |
| Mm.272548  | 2.82 | 4.37E-04| Etnk1       | Ethanolamine kinase 1 |
| Mm.197552  | 2.74 | 1.10E-03| Tgfb1       | Transforming growth factor, beta receptor 1 |
| Mm.262330  | 2.66 | 1.54E-02| Stk3        | Serine/threonine kinase 3 (Ste20, yeast homolog) |
| Mm.295263  | 2.65 | 1.43E-03| Dcamkl1     | Double cortin and calcium/calmodulin-dependent protein kinase-like 1 |
| Mm.222178  | 2.57 | 2.58E-04| Prkca       | Protein kinase C, alpha (Prkca), mRNA |
| Mm.310973  | 2.56 | 5.12E-04| Dyrk1a      | Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a |
| Mm.313594  | 2.50 | 5.38E-03| Mpp5        | Palmitoylated 5 (MAGUK p55 subfamily member 5) |
| Mm.6710    | 2.49 | 3.65E-02| Rock1       | Rho-associated coiled-coil forming kinase 1 |
| Mm.440470  | 2.46 | 3.16E-03| Crk7        | CDC2-related kinase 7 |
| Mm.389061  | 2.36 | 1.34E-03| Ptk1        | PFTAIRE protein kinase 1 (Ptk1), mRNA |
| Mm.244236  | 2.34 | 8.50E-03| Pkn7        | Protein kinase N2 |
| Mm.288141  | 2.25 | 7.64E-03| Rp2h        | Retinitis pigmentosa 2 homolog (human) |
| Mm.31672   | 2.21 | 4.65E-04| Cdk6        | Cyclin-dependent kinase 6 (Cdk6), mRNA |
| Mm.215171  | 2.20 | 5.62E-04| Trpm6       | Transient receptor potential cation channel, subfamily M, member 6 |
| Mm.3994    | 2.18 | 3.98E-04| Dusp16      | Dual specificity phosphatase 16 |
| Mm.136511  | 2.17 | 5.10E-04| Tlk1        | MKIAA0137 protein |
| Mm.332231  | 2.16 | 3.01E-03| Mag2        | Membrane associated guanylate kinase, WW and PDZ domain containing 2 |
| Mm.16340   | 2.13 | 1.50E-02| Fgfr2       | Fibroblast growth factor receptor 2 |
| Mm.275839  | 2.13 | 1.00E-02| Jak2        | Janus kinase 2 |
| Mm.393114  | 2.08 | 7.62E-04| Csnkl1      | Casein kinase 1, alpha 1 |
| Mm.17918   | 2.08 | 7.94E-04| Marcksl1    | MARCKS-like 1 |
| Mm.368668  | 2.06 | 6.49E-04| Csnkl3      | Casein kinase 1, gamma 3 (Csnkl3), mRNA |
| Mm.291936  | 2.01 | 8.02E-03| Map4k5      | Mitogen-activated protein kinase kinase kinase 5 |
| Mm.35290   | -2.00 | 1.42E-02| Ripk4       | Receptor-interacting serine-threonine kinase 4 |
| Mm.36006   | -2.39 | 1.22E-03| Ak7         | Adenylate kinase 7 |
| Mm.306163  | -2.68 | 8.63E-03| Prkar1b     | Protein kinase, cAMP dependent regulatory, type I beta |
| Mm.7373    | -2.71 | 1.05E-03| Per1        | Period homolog 1 (Drosophila) |
| Mm.32831   | -2.96 | 2.47E-02| Wif1        | Wnt inhibitory factor 1 |
| Mm.44442   | -3.76 | 2.45E-04| Kndc1       | Kinase non-catalytic C-lobe domain (KIND) containing 1 |
Genes involved in lipids biosynthesis and clearance pathways were altered in type II cells from Stat3\(^{Δ/Δ}\) mice. A. Sreb1 and Sreb2 regulate genes encoding the major metabolic enzymes in the fatty acid, cholesterol and phospholipids biosynthesis. The arrow (↑) indicates that the mRNA level was induced in Stat3\(^{Δ/Δ}\) mice; (↓) indicates the mRNA level was reduced in Stat3\(^{Δ/Δ}\) mice. Gene symbols, descriptions and the expression changes are listed in panel B.
terol were down regulated, with the exception of 3-hydroxy-3-methylglutaryl-Coenzyme A synthase (Hmgcs), which was increased. HMGCS participates in other metabolic pathways, including valine, leucine and isoleucine degradation (KEGG 00280). This analysis indicates that metabolic pathways regulating fatty acid, phospholipid, and cholesterol biosynthesis were coordinately decreased after deletion of Stat3 in type II cells, supporting an important role of STAT3 in regulating lipids biosynthesis in the lung. LDL receptor (Ldlr), mediating cholesterol uptake, and ATP-binding cassette A3 (Abca3), important for phospholipid transport, lamellar body formation and pulmonary surfactant secretion in alveolar type II cells were decreased in the Stat3+/− mice [36-38]. Recent in vitro studies from our group confirmed the direct binding of SREBP1c to the Abca3 promoter (Besnard et.al, submitted for publication). On the other hand, the increased expression of Abca1, a key facilitator of cellular cholesterol and phospholipid export [39,40] and high density lipoprotein binding protein (Hdlbp), which may function in the removal of excess cellular cholesterol, suggests that cholesterol and phospholipid clearance were induced after deletion of Stat3. Adenosine kinase (Adk), was increased 8.2 fold. Cellular role of ADK in lipid metabolism is somewhat controversial [41]. Adk deficient mice developed neonatal hepatic steatosis and die within 14 days with fatty liver [42]. Increased expression of lipid export genes and decreased expression of genes mediating lipid biosynthesis likely cause a reduction of total lipids level in the type II cells of Stat3+/− mice.

Previous studies demonstrated the susceptibility of Stat3+/− mice to lung injury and death related to surfactant dysfunction [12]. Consistent with our prediction from the present mRNA microarray analysis, the saturated phosphatidylcholine (SatPC) content in bronchoalveolar lavage fluid was significantly decreased in Stat3+/− mice (Figure 2). Significantly decreased SatPC synthesis and abnormalities in lamellar body numbers and morphology were also observed in Stat3+/− mice (data not shown). Taken together, a number of genes regulating surfactant lipid homeostasis were altered in type II cells isolated from the Stat3+/− mice, consistent with biochemical, functional, and morphologic changes in the surfactant system that is exacerbated by oxidant stress [12] or expose to pathogens [13].

**Role of Akt in Stat3 regulated lipid metabolism**

Our observations support the view that the decrease in SREBP, at least in part, results in decreased expression of genes regulating lipid biosynthesis and metabolism in type II cells from Stat3+/− mice. SREBPs are master regulators of lipid metabolism. The transcriptional targets, and the pathways mediated by SREBP in liver have been well studied [43]. SREBPs are expressed in the developing lung, SREB1c increases in the developing lung concomitantly with the perinatal increase in surfactant and lipid synthesis, surfactant protein and Abca3 expression, genes critical for surfactant function.

However, the role of Stat3 in regulating SREBP and associated lipid metabolism in the lung is largely unknown. In the present analysis, we sought to identify mechanisms by which Stat3 regulates SREBPs and associated lipid biosynthesis pathways in alveolar type II cells in the lung. The regulation of SREBPs occurs at both transcriptional and post-transcriptional levels. The post-transcriptional regulation requires SCAP. Cre-mediated disruption of Scap significantly reduced Sreb1 and 2 levels as well as SREBP target gene expression in liver [44]. There are multiple potential STAT sites on the Scap promoter. Thus, Stat3 may influence lipid biosynthesis through the transcriptional regulation of Scap. At transcriptional level, nuclear hormone receptors (Nr1h2 and Nr1h3) and Pgc1-alpha and beta are known to regulate SREBPs expression in liver. Increased expression of Srebf1 and Fasn was associated with increased hepatic triglyceride content in Stat3 deficient mice [10,45]. In the present study, expression of SREBP and their downstream targets were decreased in type II cells from Stat3+/− mice (a finding that contrast
with the findings in the liver) without changes in other
known regulators such as Nr1h2, Nr1h3, Pgcl-1-alpha or
Pgcl-1-beta, indicating the likely presence of alternative reg-
ulatory mechanisms in lung cells.

We hypothesize that AKT plays an important role in Stat3
regulated SREBP expression and associated lipogenesis in
lung based on the following observations: 1) Protein and
mRNA levels of Akt were increased in cells constitutively
expressing active Stat3 and were reduced after Stat3 deple-
tion [46]. STAT3 binds to Akt1 promoter was confirmed by
ChIP assay [47], suggesting that Akt maybe a direct tran-
scriptional target of Stat3. AKT, on the other hand, inhibits
Stat3 transcriptional activity and phosphorylation [48]. Decreased Akt gene expression seen after dele-
tion of Stat3 may represent a direct effect of Stat3
deficiency or to a negative regulatory response to STAT3
deficiency, 2) AKT activation induces both Srebf1 and
Srebf2 mRNAs and proteins as well as key enzymes in the
cholesterol, fatty acid and membrane lipid biosynthesis
pathways [49], 3) Multiple lines of evidence suggest that
PI3K influences Stat3 activation. STAT3 binds directly to
the PI3K regulatory subunits [50-52]. The expression of
genes encoding for several PI3K subunits were altered
after deletion of Stat3 [see Additional file 1], supporting
the involvement of PI3K/Akt signaling in Stat3 regulated
bioprocesses in lung, and 4) Since AKT physically interacts
with FOXA2 and regulates FOXA2-dependent tran-
scriptional activity [53], the effects of AKT may be mediated, in
part, via FOXA2. FOXA2 expression was reduced more than 2
fold in cells from the Stat3 N/A mice. FOXA2 regulates lipid
metabolism in both lung and liver [54,55]. Deletion of
either Stat3 or FOXA2 resulting in decreased expression of a
number of the overlapping genes that play important roles in
surfactant homeostasis including Abca3 [55]. Thus, interactions
between AKT and FOXA2 represent another potential mechanism by which lipid metabolism
is influenced in Stat3 N/A mice.

**Stat3 influences expression of genes mediating apoptosis
and cell survival**

Many genes modulating apoptosis/cell survival were
altered in response to the deletion of Stat3 from type II
cells, including multiple Bcl-2 family members (Bcl2, Bcl2l1, Mcl1, Bcl2l11 and Bad), caspase 3 (Casp3), FADD-
like apoptosis regulator (Cflar, also known as Flipp),
mucosa associated lymphoid tissue lymphoma transloca-
tion gene 1 (Malt1), prostaglandin-endoperoxide syn-
these 2 (Ptgs2) and nuclear receptor subfamily 3, group C,
member 1 (glucocorticoid receptor, Nr3c1). In depth liter-
ature mining identified more apoptosis related genes than
did analysis by Gene Ontology annotation. As depicted in
Figure 3, our study indicates that STAT3 regulates apopto-
sis in a complex manner via processes that occur in multi-
ple intracellular locations. Thus, STAT3 appears to serve as
key regulator of apoptosis in alveolar type II cells. Among
those apoptosis related genes, Malt1, Ptgs2 and Nr3c1
were strongly induced (> 5 fold in compare with control).
MALT1 interacts with BCL10 (increased 2 fold in Stat3 N/A
mice). The formation of this complex is essential for NF-
kappaB activation that, in turn, play a role in cell survival.
IKKbeta phosphorylates BCL10 in its MALT1 interaction
domain, causing BCL10 and MALT1 to disassociate,
resulting in attenuation of NFkB signaling and cytokine
production [56,57]. PTGS2 also known as COX-2, a key
enzyme in prostaglandin biosynthesis, that is highly
expressed in alveolar type II cells. The expression of Ptgs2
is increased in epithelial tumors, including non-small cell
lung and prostate cancers via activation of the IL-6/ 
GP130/STAT3 signaling pathway [58]. This pathway
could contribute to tumor formation by promotion of
tumor cell resistance to apoptosis via inhibitor of apopto-
sis (IAP)-dependent mechanism [58,59]. Consistent with
these observations, Ptgs2, Il6st and two of the IAP family
members (Birc4 and Birc6) were correspondingly induced
in Stat3 N/A cells (5.7, 1.7, 3.2 and 2.3 fold respectively).
Nr3c1 (increased 5.1 fold) encodes a receptor for gluco-
corticoids that can act as both a transcription factor and as
a regulator of other transcription factors. STAT3 and
NR3C1 physically interact to mediate effect of glucocorti-
coid on the IL-6-mediated inflammatory response [60-
62]. NR3C1 also interacts with stress-responsive transcrip-
tion factors (Hif1a, increased 4.7 fold), mitogen activated
protein kinase 8 (Mapk8 or Jnk, increased 4.7 fold) and
tyrosine 3-monooxygenase/tryptophan 5-monooxygen-
ase activation protein, epsilon polypeptide (Ywhal,
increased 3.3 fold), a 14-3-3 family of proteins implicated
in the pathogenesis of small cell lung cancer [63,64].

STAT3 is likely to regulate apoptosis by multiple mecha-
nisms including gene transcription. Bcl-x.L is the direct
transcription target of STAT3 [65]. STAT3 can serve as an
anti-apoptotic factor by transcriptional up-regulating the
expression of Bcl-x.L [66]. The decrease of Bcl-x.L may rep-
resent a direct response to Stat3 deletion. The fact that
expression of Bcl-x.L blocked the apoptotic effects of the
adenovirus in lung injury suggested that Bcl-x.L may medi-
rate the role of STAT3 in the regulation and survival of the
respiratory epithelium [13]. The PI3k-Akt pathway rep-
resents a second mechanism by which STAT3 influences
cyteprotection. PI3k-Akt signaling mediates a wide range
of down stream targets to regulate apoptosis [67]. For
example, AKT phosphorylates multiple Bcl-2 family mem-
bers, including BAD and Bcl-x.L [68], inhibits caspase 3
activation [66] and blocks cytochrome C release from
mitochondria [69]. Another mechanism by which STAT3
modulates apoptosis is through protein-protein interac-
tions. Bcl-x.L is a direct transcriptional target of STAT3; Bcl-
x.L interacts with VDAC1 to regulate the outer mitochon-
drial membrane channel induce apoptosis [70,71].
CASP3 can interact with multiple apoptosis proteins including CFLAR, BIRC4 and 6, BCL2 and APP [72-75]. The expression of Casp3 as well as its interaction partners was induced in Stat3Δ/Δ mice. NR3C1 has both pro- and anti-apoptotic effects. NR3C1 physically interact with STAT3, HIF1A, MAPK8, YWHAL [63,64,76]; these stress-responsive transcription factors and signaling molecules were largely induced in the present array from Stat3Δ/Δ type II alveolar epithelial cells. The close transcriptional communication and physical interactions among these transcriptional regulators likely play a critical role in regulating the balance of apoptosis and cell survival. In the present study, effects of STAT3 deletion were assessed in type II epithelial cells purified from the adult mouse lung. mRNA was isolated immediately after isolation to avoid cell culture dependent alteration in gene expression. It is possible that the cells have undergone added cellular stress during protease treatment, isolation and purification, which in turn may influence the expression of genes. Our results support the view that STAT3 regulates the balance between a subset of pro- and anti-apoptotic genes, determining the cell death or survival through multiple mechanisms. Consistent with the present microarray prediction, cleaved caspase-3 and TUNEL positive cells were...
significantly increased in Stat3Δ/Δ mice following adenoviral infection and the apoptosis can be blocked by expression of Bcl-xL [13].

**Conclusion**
Our previous studies demonstrated that Stat3 plays critical role in cyto-protection during lung injury [12,13]. Present data support the role of Stat3 in enhancing epithelial cell survival and surfactant lipid synthesis that contribute to the maintenance of lung function. Deletion of Stat3 from type II alveolar epithelial cells induced the expression of the genes regulating protein metabolism, protein transport, chemotaxis and apoptosis while decreasing the expression of genes regulating lipid synthesis and metabolism. Critical to pulmonary function during injury, Stat3 influences the expression of genes regulating surfactant lipid synthesis and surfactant homeostasis including Abca3. As illustrated in Figure 4, the present study identified a complex regulatory network by which Stat3 regulates gene expression in type II alveolar cells that is required for cellular homeostasis following injury. STAT3 likely interacts with AKT/FOXA2 in the regulation a number of biological processes in alveolar type II cells, including cell survival/apoptosis, cholesterol and fatty acid biosynthesis required for surfactant homeostasis and lung function.

**Methods**

**Transgenic Mice**
SP-C-rtTA/(tetO)7CMV-Cre/Stat3flx/flx triple-transgenic mice were generated as described previously [12]. Stat3flx/flx mice were a kind gift of Dr. Takeda (Hyogo College of Medicine, Japan) [8]. In the presence of doxycycline, exon 21 of the Stat3 gene is permanently deleted from respiratory epithelial cells prior to birth (Stat3Δ/Δ mice) [12]. Stat3 deleted transgenic (Stat3Δ/Δ) and non-deleted litter-

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**Figure 4**
Schematic representation of the proposed role of Stat3 in cytoprotection of the lung in normal (A) versus Stat3Δ/Δ mice (B). Stat3 plays critical role in cyto-protection during lung injury [12, 13]. Present data support the role of Stat3 in enhancing epithelial cell survival and surfactant lipid synthesis that contribute to the maintenance of lung function. For simplicity, only representative genes were included.
mates (double transgenic, SP-C-rtTA, or tetO7CMV-Cre and Stat3Δ/Δ) were used for the experiments. Doxycycline was administered to the dams in the food at a concentration of 625 mg/kg (Harlan Teklad, Madison, WI) from embryonic day 0 (E0) to postnatal day 25 (P25), resulting in extensive deletion of Stat3 in respiratory epithelial cells [12]. As previously described, deletion of Stat3 did not alter lung size, morphology or survival under non-stressed condition [13].

**RNA Extraction**

Alveolar type II cells were isolated from 8 weeks old, sex and age matched littermate control and Stat3Δ/Δ mice using collagenase and differential plating as described by Rice et al. [77]. Type II cells from 3 mice were pooled to obtain one cell pellet. Three independent pools were generated from control and Stat3Δ/Δ mice separately for purification of RNA and microarray hybridization. Type II cells were homogenized with TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentration was measured by spectrophotometer and normalized prior to cDNA synthesis. RNA was extracted from alveolar type II epithelial cells isolated from Stat3Δ/Δ mice separately for purification of RNA and microarray hybridization. These cell isolates consist of more than 90% alveolar type II cells with residual alveolar macrophages as the major contaminating cell. Purity was assessed by modified Papanicolaou stain. Purity and number of type II cells isolated from Stat3Δ/Δ mice were not different from controls.

**RNA Microarray Analysis**

mRNA was extracted from three independent pools of isolated type II cells from adult Stat3Δ/Δ and control mice. The cRNA was then hybridized to the murine genome MOE430 (consists of ≈ 45000 gene entries) chips (Affymetrix, Santa Clara, CA) according to the manufacturer’s protocol. The RNA quality and quantity assessment, probe preparation, labeling, hybridization and image scan were carried out in the CCHMC Affymetrix Core using standard procedure. RNA quality and quantity were analyzed by spectrophotometer. The A260/A280 ratio was used to determine RNA purity with the acceptable region of 1.9–2.1. Affymetrix Microarray Suite 5.0 was used to scan and quantitate the gene chips under default scan settings. Normalization was performed using the Robust Multichip Average model [78,79]. Data were further analyzed using affyLimGUI from R/Bioconductor package [80]. Differentially expressed genes were selected with the threshold of T-Test P-value ≤ 0.05, False Discovery Rate (FDR) ≤ 10% and fold change ≥ 1.5. We prioritized the mRNAs whose abundance consistently changed in multiple probe sets by selecting them without the FDR consideration. Unknown cDNA clones/ESTs and duplicated gene entries were removed from further functional analysis.

**Gene Ontology Analysis**

Gene Ontology Analysis was performed using public available web-based tool David (database for annotation, visualization, and integrated discovery) [81]. Overrepresented biological processes were selected at the threshold of Fisher Exact Test P-Value ≤ 0.05 and minimum gene counts belonging to an annotation term ≥ 2%.

**Promoter Analysis: Transcription Factor Binding Sites Search**

Promoter sequences of all differentially expressed genes (-2000 bp upstream from the tentative TSS) were retrieved from our database (originally downloaded from UCSC Genome browser) and searched for over-represented TFBS (Transcription Factor Binding Site) in these sequences using MatInspector (Genomatix) using the complete Vertebrate Matrix Library 6.2. The P value is calculated using binomial distribution probability by comparing the matrix match of the promoter regions of differentially expressed genes with the promoters from random mouse genes sets (-2kb for all the promoters). The single-step Bonferroni adjustment is used to control for the multiple comparison effect (i.e., multiplication of p-value by the number of TFBS in Genomatix Vertebrate Matrix Library).

**Pathway Enrichment Test**

Overrepresented pathways were identified by comparison the overlap of differentially expressed genes and all genes in MOE430 mouse genome (reference) with the known KEGG pathways. A Fisher’s exact test for 2×2 contingency was used to calculate the statistical significance. A pathway is considered to be over-represented when a probability P value = 0.01 and gene frequency (genes in the pathway/total number of differentially expressed genes) ≥ 2%.

**Literature Mining**

Potential protein-protein or protein-DNA interactions were identified using Pathway Studio (Ariadne Genomics) that contains MedScan, an automated text-mining tool to search the entire PubMed and other public sources (The current version database contains more than 15 million Medline abstracts/full text). Gene expression profiling results was imported into the Studio and used to interpret pathways, gene regulation networks, and protein interaction maps.

**Validation of mRNAs**

Real-time RT-PCR was used to cross validate changes in a subset of genes from microarray selection. mRNA was extracted from alveolar type II epithelial cells isolated from Stat3Δ/Δ and control mice using RNAeasy Protect mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. RNA concentration was measured by spectrophotometer. cDNA was made with SuperScript
First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Maltl1, Rtn4, Reg3g, Bcl2l1, Abca3, Scap, Fasn, Sreb1f and Sreb2f were detected using primers listed Changes in mRNA were determined in type II cells isolated from Stat3+/Δ mice and controls (n = 3–4/group). The following primers were used: Maltl1: forward, TAT CCA GGA GGA CCC CAT GT and reverse, TCT GAT CAT TTA GCA TCA G and reverse, AGG ACC CAA AAC CGC TGT GTC CAG; and reverse, TGG CAC AGA CGA TGA C and reverse, GTC GAA CTT TGA CCA CAA ACA AGG AGA GC and reverse, CAG reverse, TCC GGC CAT CCT CAG TGG TGG G; Bcl2l1: forward, TCT CTC TCC TCT GTG CAT CCT and reverse, GCC CAT CCT CAG TGG TGG G; Scap: forward, TGA CCA ACA AGA AGG AGA GC and reverse, CAG GAA CAC CAA ACA AGA AGG GC; Sreb1f: forward, AAG CCG GGT GGG CGC CGG CGG CAT and reverse, GTC GTT CAA AAG AGC CCT CAG GA; Sreb2f: forward, CAT CCA GGA GCC TTT GAT ATA CCA G and reverse, AGG ACC GGG TCC TGC ACC TGT G. Fasn: forward, GGA CAT GGT CAC AGA CGA TGA C and reverse, GTC GAA CCT GAG ATC TTC CIT CA. The PCR product was separated by the 1% agarose gel electrophoresis. b-actin was used as the internal control. Taqman Gene Expression Assay were used to confirm the expression ofAkt2, Cdipt, Acox2, Cad2 and Gpam using Applied Biosystems 7300 Real-Time PCR System and company designed probes (Applied Biosystems). Statistical differences were determined using unpaired Student’s t-tests.

Acknowledgements

We thank Ann Maher for secretarial assistance, Valerie Besnard for scientific discussion. We also thank Dr. K. Takeda (Hyogo College of Medicine) for the gift of Stat3fxf/fx mice.

This work was supported by National Institutes of Health Grant HL61646 to M. Ikegami, Y. Xu and J.A. Whitsett.

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