Peroxisome Proliferator-activated Receptor \(\gamma\) (PPAR\(\gamma\)) Mediates a Ski Oncogene-induced Shift from Glycolysis to Oxidative Energy Metabolism*§‡

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Background: Oncogenic transformation is usually accompanied by increased aerobic glycolysis, lipid metabolism, and glutamine catabolism.

Results: Ski-transformed fibroblasts shift from aerobic glycolysis to oxidative metabolism of fatty acids, mitochondrial biogenesis, and glutamate catabolism.

Conclusion: The Ski-induced metabolic alterations require the activity of the lipid-activated transcription factor PPAR\(\gamma\).

Significance: The metabolic program accompanying oncogenesis may be more varied than currently appreciated.

Overexpression of the Ski oncogene induces oncogenic transformation of chicken embryo fibroblasts (CEFs). However, unlike most other oncogene-transformed cells, Ski-transformed CEFs (Ski-CEFs) do not display the classical Warburg effect. On the contrary, Ski transformation reduced lactate production and glucose utilization in CEFs. Compared with CEFs, Ski-CEFs exhibited enhanced TCA cycle activity, fatty acid catabolism and glucose utilization in CEFs. Compared with CEFs, Ski-CEFs (Ski-CEFs) do not display the classical Warburg effect. On the contrary, Ski transformation reduced lactate production and glucose utilization in CEFs. Compared with CEFs, Ski-CEFs exhibited enhanced TCA cycle activity, fatty acid catabolism and glucose utilization in CEFs.

The nuclear oncoprotein Ski is a bifunctional transcription factor that either activates or represses transcription. Ski does not bind DNA directly (1) but interacts with several DNA-binding transcription factors to regulate gene expression. In several instances, this mechanism, first described in studies with avian embryo fibroblasts, has been predictive of the role of Ski in mammalian development and oncogenesis. Notably, interaction of Ski with the TGF-\(\beta\)-activated Smad proteins results in transcriptional repression and is correlated with its oncogenic transformation of CEFs (2–4). Suppression of TGF-\(\beta\) signaling by this mechanism mediates the oncogenic activity of SKI in several human cancers (5–7). Ski also functions as a transcriptional co-repressor with GATA1, PU.1, and the retinoic acid receptor in suppressing differentiation and promoting oncogenesis of human hematopoietic cells (8–10). The action of Ski as a retinoic acid receptor co-repressor was first described in studies of its action in avian cells (11).

In apparent contrast to its oncogenic activity in avian embryo fibroblasts, Ski was found to induce skeletal muscle differentiation in these cells (12). This in vitro activity correctly predicted a role for Ski in mammalian muscle differentiation in vivo and in vitro (13, 14). In this case, Ski functions as a transcriptional co-activator in a complex with Six1 and Eya3 (14). Ski knockout mice display a dramatic reduction of skeletal muscle development (15), as well as the virtual absence of brown adipose tissue (16). Transgenic mice overexpressing c-Ski exhibit a remarkable increase of skeletal muscle mass but a decrease of adipose tissue (17). In these mice, selective hypertrophy of type Iib fast skeletal muscle fibers is accompanied by increased lipid oxidation despite decreased expression of the transcription factor peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)), a key regulator of lipogenesis (17). These findings suggest that Ski plays a role in regulating lipid metabolism, perhaps mediated by an effect on the expression or transcriptional activity of PPAR\(\gamma\).

PPAR\(\gamma\) is a lipid ligand-activated nuclear receptor that regulates adipogenesis and has a complex role in lipid metabolism (18–20). Several genes involved in lipid uptake, intracellular

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transport, synthesis, and β-oxidation are transcriptionally activated by PPARγ (21–24). PPARγ also induces expression of its co-activator, PGC1-α, which stimulates fatty acid oxidation, mitochondrial biogenesis, and cell respiration in muscles and brown adipose tissue (25–27). These data indicate that PPARγ plays an important role in cellular metabolism, promoting not only lipid transport, storage, and lipogenesis but also lipid oxidation.

Altered cellular metabolism is a hallmark of oncogenic transformation. In general, cancer cells exhibit the classical Warburg effect of dramatically accelerated glycolysis under aerobic conditions (28). This metabolic change also is noted in fibroblasts transformed by oncogenes such as γ-Src (29, 30). These cells rapidly acidify cell culture medium due to the accumulation of lactate generated by glycolytic conversion of glucose. Primary CEFs, transformed by overexpression of Ski, exhibit many features common to oncogenic transformation (31), but surprisingly they acidify the culture media very slowly even after reaching confluence (32). It therefore seemed likely that Ski-CEFs produce much less lactate and therefore rely less on glycolysis than CEFs. This possibility taken together with results obtained from analyses of Ski transgenic and Ski−/− mice led us to investigate whether Ski might regulate metabolic energy production, perhaps by increasing lipid metabolism in concert with PPARγ. Here, we demonstrate that Ski has a profound effect on cellular energetics by decreasing the reliance on glycolysis and stimulating biogenesis of mitochondria and lipid oxidation.

EXPERIMENTAL PROCEDURES

Cell Culture and Retroviral Transduction—Primary CEFs were prepared and cultured as described previously (31), and CEFs were transformed by retroviral overexpression of c-Ski in RCAS-Ski (13). PPARγ expression was ablated using a replication-defective retroviral vector that expresses shRNAs in the framework of microRNA-30 as well as the puromycin resistance gene (supplemental Fig. S3). Versions of this vector, RdpImiR30shRNA, expressing four different PPARγ-targeted shRNAs were co-transfected with RCAS-Ski using FuGENE 6 (Roche Applied Science) into CEFs. Seventy two hours after transfection, cells were selected with puromycin (4 μg/ml, Sigma). Survivors were cloned in 50% conditioned medium and screened by Western blotting to assess PPARγ knockdown.

Lactate and Glucose Assays—Medium was sampled every 4 or 24 h from cultured CEFs, Ski-CEFs, or PPARγ knockdown Ski-CEFs (PPARγKD-Ski-CEFs). Harvested medium was diluted 50 times for lactate measurement and 100 times for glucose measurement. The concentration of lactate in the medium was determined enzymatically using a spectrophotometric method (33). The glucose assay was done according to the instructions of the glucose assay kit (Sigma).

Metabolic and Mitochondrial Activity Assays—Oxygen consumption was measured with an Oxygraph-2K (OROBOROS Instruments). For intact cell respiration, cells were trypanized, counted, and resuspended in growth medium at a concentration of 1 × 10⁶/ml. For respiration in permeabilized cells, the cells were resuspended at the same concentration in Mir05 respiration medium (34). The optimal concentration of digitonin (1.2 μg/ml) required to permeabilize cells was determined in a pilot experiment. Mitochondrial respiration was examined using specific substrates and inhibitors according to protocols described previously (35). Heat generation of CEFs and Ski-CEFs (1 × 10⁶/1.4 ml) was measured by isothermal calorimetry (Microcal), and growth medium alone was measured as background. The reference power value (30 μcal/s) and cell number were optimized by pilot experiments. Citrate synthase activity was measured as described previously (36). To measure AlamarBlue® reduction, CEFs or Ski-CEFs (2 × 10⁴/well) were plated in 96-well plates. Six hours after plating, cells were provided with fresh growth medium containing 10% alamarBlue® (Invitrogen). The plates were read at 590 nm for fluorescence intensity every 60 min. Data were normalized to total protein and subjected to linear regression analysis.

Flow Cytometry—Cells were suspended in growth medium at a concentration of 2 × 10⁶/ml, stained with MitoTracker Green (MTG, 80 nM, Invitrogen) or MitoTracker Red (MTR, 100 nM, Invitrogen) for 30 min at 37 °C. Cells were then briefly washed with pre-warmed medium and immediately analyzed using an SLR II flow cytometer (BD Biosciences). Data collected from 10,000 cells were analyzed by Flowjo.

Electron Microscopy—CEFs, Ski-CEFs, and PPARγKD-Ski-CEFs (2 × 10⁵ cells/ml) were fixed with glutaraldehyde (2.5%), stained with uranyl acetate (0.25%), and embedded using a minor modification of the previously described methods (37). Thin sections (70 nm) were cut with an RMC MT6000-XL ultramicrotome, carbon-coated with a Denton DV-401 (Denton Vacuum), and examined with a JEOL 1200EX electron microscope.

Mass Spectrometric Measurement of Deuterium Incorporation into Palmitate and Triglyceride—To measure stable isotope incorporation, cells at 80–90% confluence were switched into fresh medium containing 10% ²H₂O and cultured at 37 °C for 24 h. Medium was removed and saved for percent enrichment measurements, and the cells were isolated and stored at −80 °C until analysis. Triglyceride concentrations and de novo lipogenesis were measured by mass spectrometry by the Case Mouse Metabolic Phenotyping Center as described by Bederman et al. (38). The ²H label on triglyceride-derived glycerol and palmitate measured the amount of newly synthesized triglyceride and palmitate, respectively, which were calculated as described previously (39, 40).

Quantitative Reverse Transcription-PCR (q-RT-PCR)—RNA isolation, cDNA synthesis, and real time PCR using the iCycler real time PCR detection system (Bio-Rad) were performed as described previously (14). The fold change in expression of each mRNA relative to ATPB3 was calculated as 2−ΔΔCT, where ΔCT = CTtarget − CATPB3 and ΔΔCT = ΔCTCEF − ΔCTCEF or ΔCTCEF − ΔCTPPARγKD.

Western Blotting—Whole-cell extracts were prepared and immunoblotted as described previously (14). The monoclonal antibodies (mAb) used were as follows: Ski (G8 (41), Lerner Research Institute Hybridoma Core Facility), PPARγ (E8, Santa Cruz Biotechnology), MFN2 (XX-1, Santa Cruz Biotechnology), CYC (6H2, monoclonal, Santa Cruz Biotechnology), β-actin (Sigma), NDUFS3 (MS112), 70-kDa Fp (MS204), PDH1α (MSP07), complex III subunit core1 (MS303), and porin (MSA03). Antibodies for the last five mitochondrial proteins
are all from Mitosciences. Densitometry was analyzed by ImageJ (National Institutes of Health).

**Metabolite Oxidation**—10^6 cells were suspended in 3 ml of growth medium in a flask that contained [1-14C]acetate (5 mM, 1 μCi), [U-14C]glucose (30 mM, 1 μCi), [1-14C]glutamate (1 mM, 0.5 μCi), [1-14C]octanoate (0.1 mM, 0.5 μCi), or [1-14C]palmitate (0.1 mM, 0.5 μCi) and incubated for 2 h at 37 °C. The experimental protocol and devices used for trapping released 14CO2 were described previously (42). Measurements of the 14C in the medium and 14CO2 were obtained by scintillation counting (Beckman Coulter).

**Reporter Assay**—2 × 10⁴ cells (CEF and Ski-CEF) were plated per well of a 96-well plate and transfected with 20 ng of p-CMV-PPRE firefly luciferase reporter and 20 ng of pGL3tk Renilla luciferase reporter in the presence or absence of Ski expression plasmid. Twelve hours later, cells were treated with or without 10 μM GW1929. Luciferase activity was measured 48 h after transfection according to the protocol for the Dual-Glo luciferase assay system (Promega). For each experiment, at least six replicates were performed.

**Statistical and Data Analysis**—Graphs were drawn and related statistical analyses were performed using GraphPad Prism5 (GraphPad software). For data comparisons involving multiple variables, two-way analysis of variance was used. For comparisons involving two variables, we used Student’s t test. Expression array data were normalized and analyzed by GeneSpring (Agilent Technologies) followed by ontological analysis using Ingenuity Pathway Analysis (Ingenuity Systems).

**RESULTS**

**Glycolysis Is Suppressed in Ski-CEFs**—To establish the basis for the reduced acidification of culture medium by Ski-CEFs as compared with that of CEFs, we measured lactate production by both cell types during 24 h after medium change and during a 5-day time course. The resulting data show that Ski-CEFs produced about half as much lactate as CEFs during the initial 24-h period (Fig. 1A) and accumulated less than one-fifth the lactate compared with CEFs in 5 days (Fig. 1B). To confirm these findings, we next examined the consumption of glucose, the major source of lactate in the culture medium. As expected, we found that, compared with CEFs, Ski-CEFs utilized two-thirds as much glucose at 24 h and one-fifth as much in 5 days (Fig. 1, C and D).

Reduced lactate production and glucose utilization indicated that Ski caused a decreased rate of glycolysis in CEFs. However, it did not seem likely that this reflected an overall reduction in metabolic activity, because Ski-CEFs appear to be the same size and to proliferate at the same rate as CEFs (12). To assess the metabolic activity of these cells, we measured the reduction of alamarBlue, which is often used to assess the activity of mitochondrial NADH dehydrogenase (43). Because alamarBlue is reduced by cellular dehydrogenases that utilize FAD, NAD, and NADP as coenzymes (44, 45), its reduction reflects the overall metabolic state of cells (46). Here, we show that the rate of alamarBlue reduction by Ski-CEFs is at least 1.8-fold more than that of equal numbers of CEFs (Fig. 1E).

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**TCA Cycle Flux Is Enhanced in Ski-CEFs**—Their decreased rate of glycolysis and increased overall metabolic activity suggested that Ski-CEFs have a higher rate of tricarboxylic acid (TCA) cycle flux than CEFs. We thus examined this possibility by measuring 14CO2 production from [1-14C]acetate, [U-14C]glucose, and [1-14C]glutamate (Fig. 2). The results demonstrated that flux through the TCA cycle was accelerated in Ski-CEFs.
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CEFs compared with that of CEFs (Fig. 2, A–C). Acetate, which enters the TCA cycle directly as acetyl-CoA, was oxidized 4.9 times faster. Glucose carbon, which enters the TCA cycle as acetyl-CoA via pyruvate dehydrogenase complex, was oxidized 3.5 times faster. Although they consumed much less glucose in 24 h than CEFs, about 10% of the total glucose utilized by Ski-CEFs was oxidized by the TCA cycle, whereas this ratio was about 2% in CEFs (Figs. 1C and 2B). Glutamate, which enters TCA cycle as α-ketoglutarate, was oxidized to CO2 6.7-fold faster by Ski-CEFs than CEFs (Fig. 2). Taken together, our data indicate that the overall rate of TCA cycle flux is about five times greater in Ski-CEFs than in CEFs.

Ski-CEFs Exhibit an Enhanced Rate of Mitochondrial Respiration—The combined results above suggested that Ski-CEFs have a high level of mitochondrial respiration. Two methods were used to measure mitochondrial respiration, heat generation and oxygen consumption. Isothermal calorimetry was used to measure heat production, which is a side product of cytochrome oxidase activity, the final electron acceptor of the electron transport chain (ETC). Ski-CEFs generated 2.6-fold more heat than CEFs, suggesting that a higher fraction of respiration might be uncoupled from oxidative phosphorylation in Ski-CEFs (Fig. 3A). To examine this possibility and to assess mitochondrial function in more detail, we measured oxygen consumption by intact Ski-CEFs and CEFs in the absence and presence of the ATP synthase inhibitor, oligomycin, and the uncoupling agent, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). We found that constitutive (routine) oxygen consumption of Ski-CEFs was about 2-fold greater than that of CEFs (Fig. 3, B and C). Upon treatment with oligomycin, this rate was reduced by an even greater extent in Ski-CEFs than in CEFs, indicating that oxygen consumption was coupled to ATP generation. The maximal uncoupled respiration of Ski-CEFs was 3-fold higher than that of CEFs, providing evidence that Ski-CEFs have greater oxidative capacity. The low level of residual oxygen consumption in both cell types after inhibition of complex III with antimycin A shows that the measured oxygen consumption was due almost completely to mitochondrial respiration. These results demonstrated that Ski-CEFs have not only a higher rate of respiration that is coupled to ATP synthesis but also greater overall oxidative capacity than CEFs.

To determine the basis of enhanced respiration, we measured oxygen consumption by intact mitochondria of permeabilized cells in the presence of selective substrates and inhibitors (Fig. 3D). Glutamate and malate provided electrons via NADH to complex I of the ETC. Upon cell permeabilization, the rate of oxygen consumption decreased to a very low level because of depletion of endogenous ADP. This so-called “leak” indicated that only a minor proportion of oxygen consumption by mitochondria of both cell types was unrelated to oxidative phosphorylation. The stimulation of oxygen consumption by ADP in both cell types demonstrated a tight coupling of electron transport to ATP synthesis. Moreover, ADP addition increased respiration of Ski-CEF mitochondria to a much higher level than that of mitochondria from CEFs. By adding succinate to glutamate plus malate, electrons were fed simultaneously into complexes I and II. This resulted in a similar fold increase in the rate of respiration in both cell types, so that Ski-CEFs maintained the 3-fold higher respiration rate that was observed prior to succinate addition. Consistently, oxygen consumption in the maximally uncoupled state following FCCP titration was much greater in Ski-CEFs than in CEFs. Subsequently, inhibition of complex I by rotenone allowed measurement of complex II-dependent respiration only, which was also greater in mitochondria from Ski-CEFs. Mitochondria from both cell types showed virtually no basal respiration following inhibition of complex III by antimycin A. Finally, measurement of complex IV respiration by the addition of ascorbate and N,N,N’,N’-tetramethyl-p-phenylenediamine showed a similar difference between the two cell types, as observed for complexes I and II.

Quantification of oxygen consumption showed that the rate of respiration by permeabilized cells had the same pattern as that of intact cells (Fig. 3E). Routine oxygen consumption by the mitochondria of Ski-CEFs was 2-fold higher compared with those of CEFs. When maximally uncoupled by FCCP treatment, permeabilized Ski-CEFs consumed 3-fold more oxygen than CEFs, which also was consistent with the intact cell measurements. Respiration with substrates feeding electrons into either complex I, complex II, or complex IV was increased about 3-fold in Ski-CEFs, as compared with CEFs. Thus, the enhanced oxygen consumption caused by Ski was due to a similar increase in activity of all ETC complexes and not to a selective effect on a specific ETC complex. In combination, the results demonstrate that Ski-CEFs had an enhanced mitochondrial oxidative capacity, which must have resulted from an increase in the number or mass of mitochondria.

**FIGURE 2.** Ski stimulates TCA cycle activity in CEFs. TCA cycle activity was assessed by measuring the oxidation of [1-14C]glucose; B, [U-14C]glutamate; C, [1-14C]glutamate. Values are means ± S.D. A, **p < 0.01; B, ***p < 0.0001; C, *p < 0.05.

A

B

C

Ski-CEFs have a high level of mitochondrial respiration.
Ski Increases Mitochondrial Number and Mass in CEFs—To determine whether Ski-CEFs have more mitochondria than CEFs, we first examined mitochondrial protein expression by immunoblotting. We found that expression of mitochondrial outer membrane proteins (MFN2 and porin), mitochondrial intermembrane space protein (CYC), mitochondrial matrix protein (PDHE1α), and mitochondrial ETC proteins (complex I subunit NDUF53, complex II subunit SDHA, and complex III subunit UQCRC1) was greater in Ski-CEFs as compared with CEFs (Fig. 4A). In addition, assays of citrate synthase activities also indicated that the mitochondrial content of Ski-CEFs is greater than that of CEFs (Fig. 4B). This conclusion was confirmed by both flow cytometric and electron microscopic analyses. We analyzed CEFs and Ski-CEFs by flow cytometry, using both MTG and MTR, fluorescent dyes that covalently bind mitochondrial matrix proteins irrespective of mitochondrial membrane potential (47). The observed increased fluorescence of Ski-CEFs compared with that of CEFs indicates a 2.2-fold greater mitochondrial mass per cell (Fig. 4C). Forward scattering data (not shown) indicated that the increased mitochondrial mass per cell was not due to a difference in cell size between Ski-CEFs and CEFs. Finally, electron microscopy (EM) indicated that both mitochondrial number and size were increased in Ski-CEFs (Fig. 4D). We also assessed the number of mitochondria per cell by quantifying mitochondrial genomic DNA (mtDNA) by real time q-PCR (48). Our results, normalized to a single copy nuclear gene, showed that these two cell types have the same amount of mtDNA per cell (supplemental Fig. S1). These results contrasted sharply with the data obtained with four other methods (Fig. 4), which clearly demonstrated that Ski-CEFs contain much larger and about 2-fold more mitochondria than CEFs. This increase in mitochondrial mass without a commensurate increase in mitochondrial genomes is likely due, in part, to differences in the number of genomes per mitochondrion in the two cell types (49).

Expression of PPARγ and Genes Involved in Lipid Metabolism That Are Regulated by PPARγ Are Increased by Ski—To determine the molecular basis for the increased mitochondrial function and mass in Ski-CEFs, we explored global mRNA expression by Affymetrix microarray. Analysis of the results using GeneSpring and Ingenuity Pathway Analysis software indicated that a cluster of 203 genes involved in lipid or glutamate metabolism were up- or down-impacted by Ski (supplemental Table S1). These genes included the lipid metabolism and mitochondrial biogenesis regulator, PPARγ, which is elevated by 4.4-fold in Ski-CEFs. Furthermore, several of these induced genes are known to be directly regulated by PPARγ, including CD36, ACOX1, FABP1, -3, and -4, FATP-1, Gyk, Lpl, and Perilipin 1 (21). We confirmed by q-RT-PCR and immunoblotting that PPARγ was dramatically up-regulated at both the mRNA and protein levels in Ski-CEFs. Interestingly, the expression of PPARα and PPARδ is not significantly different between Ski-CEFs and CEFs (Fig. 5, A and B). q-RT-PCR data also verified the increased expression of PPARγ-regulated genes involved in

**FIGURE 3. Analysis of mitochondrial activity.** A, heat production in growth medium was measured by isothermal calorimetry. n = 8. B, representative trace of oxygen consumption by intact CEFs and Ski-CEFs in growth medium treated sequentially with oligomycin (2 μg/ml) = leak, FCCP = uncoupled, and antimycin A (Antm A 2.5 μM) = residual oxygen consumption (ROX). C, oxygen consumption in each state (B) represented as bar graphs. Routine = untreated cells; n = 12. D, representative trace of oxygen consumption by permeabilized CEFs and Ski-CEFs in Mito05 respiration medium. The ETC states and complexes indicated at the top of the panel were assessed by sequential addition of glutamate (10 mM) and malate (2 mM), digitonin, ADP (2.5 mM), succinate (10 mM), FCCP, rotenone (Rot, 0.5 μM), antimycin A (Antm A 2.5 μM), ascorbate (As, 2 mM) plus N,N,N′,N′-tetramethyl-p-phenylenediamine (0.5 mM), and sodium azide (Azd, 100 mM). E, equilibrium values of oxygen consumption of permeabilized cells in all states shown in D are summarized as bar graphs. Routine = prior to treatment. Leak = after permeabilization; complex I = after ADP addition; complex I and complex II = after succinate addition. Uncoupled = maximum effect of FCCP; complex II = after rotenone, complex IV = ascorbate + N,N,N′,N′-tetramethyl-p-phenylenediamine minus sodium azide. The ROX value was subtracted from all other values as background. n = 6. Values are means ± S.D.; *p < 0.05; **p < 0.01; ***p < 0.001.
lipid transport and metabolism, as well as, PGC-1α, a gene related to mitochondrial biogenesis (Fig. 5C). These gene expression data suggest a strong connection between PPARγ activation and the alteration in cellular energy metabolism affected by Ski.

**Ski is a Co-activator of PPARγ-dependent Transcription**—In light of these findings, we examined the functional significance of elevated PPARγ expression more directly by performing transient transcription assays using a luciferase reporter controlled by a PPARγ-responsive element (PPRE-luc). The results showed that under normal growth conditions, with no added PPARγ ligand, the reporter was 5-fold more active in Ski-CEFs than in CEFs (Fig. 5D). In both cell types, activity was stimulated by treatment with the PPARγ ligand GW1929. Importantly, the results of co-transfection reporter assays in CEFs indicated that Ski dramatically transactivated PPRE-luc (Fig. 5E). Expression of the reporter was induced about 5-fold by addition of GW1929, and co-transfection of Ski increased ligand-activated transcription by 20-fold. These results suggested that Ski acts as a co-activator by direct interaction with PPARγ. This suggestion was supported by the finding that endogenous PPARγ co-immunoprecipitated with Ski (Fig. 5F). It is thus likely that PPARγ signaling is strongly activated by Ski, which is responsible for the elevated expression of PPARγ targets leading to enhanced lipid metabolism in Ski-CEFs.

**Ski Increases β-Oxidation of Fatty Acids**—To determine whether the observed changes in PPARγ-regulated gene expression were reflected in alterations of lipid metabolism, we measured fatty acid oxidation in Ski-CEFs and CEFs (Fig. 6, A and B). We found that oxidation of both medium chain (octanoate) and long chain (palmitate) fatty acids was about two times greater in Ski-CEFs than in CEFs. These results suggest that the observed enhancement of TCA cycle flux in Ski-CEFs is in part driven by the increased acetyl-CoA produced by fatty acid β-oxidation. Although it seemed unlikely, because it would result in a futile cycle, we investigated whether increased lipid synthesis might provide fatty acids for enhanced β-oxidation in Ski-CEFs by measuring the incorporation of 2H2O into palmitate and triglycerides by mass spectrometry (Fig. 6, C and D). Not surprisingly, we found that the contribution of de novo lipogenesis to newly synthesized palmitate was 60% less in the Ski-CEFs compared with CEFs. In addition, by measuring 2H-labeled triglyceride-glycerol, we found that the amount of newly made triglyceride was similar in CEF and Ski-CEF cells. Given the observation of decreased palmitate synthesis but increased palmitate oxidation in Ski-CEFs, we concluded that increased utilization of fatty acids rather than de novo synthesis is responsible for the metabolic shift to more oxidative metab-

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**FIGURE 4.** Ski increases mitochondrial mass and numbers. A, immunoblot of mitochondrial proteins in CEFs and Ski-CEFs. MFN2 = mitofusin-2; PDHE1α = pyruvate dehydrogenase subunit; CYC = cytochrome c; NDUFS3 = NADH dehydrogenase (ubiquinone); SDHA = succinate dehydrogenase A; and UQCRC1 = ubiquinol-cytochrome c-reductase complex core protein 1. β-Actin served as a loading control. B, activity of citrate synthase was measured with lysates from 1 × 10^6 cells. Values are means ± S.D., n = 5, ***, p < 0.001. C, CEFs and Ski-CEFs were stained with MTG or MTR and analyzed by flow cytometry. D, electron micrographs of CEFs and Ski-CEFs at the indicated magnifications. Scale bars, 2 μm at 3000×, 1 μm at 10,000×, and 0.5 μm at 60,000×.
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This conclusion was further supported by our microarray and q-RT-PCR data showing an increased expression of several genes involved in the import, oxidation, and intracellular transport of fatty acids in Ski-CEFs.

PPARγ Mediates the Induction of Oxidative Metabolism and Mitochondrial Mass by Ski—To determine whether PPARγ is directly involved in the Ski-induced enhancement of mitochondrial mass and lipid oxidation, we used RNAi to knock down expression of the endogenous PPARγ in Ski-CEFs. We screened and selected Ski-CEF clones with optimal PPARγ ablation by three different shRNAs (Fig. 7A). Although PPARγ expression was not completely eliminated by any of these shRNAs, it was reduced to levels at or below that of CEFs. The metabolic activity of these clones was then compared with that of Ski-CEFs and CEFs by first measuring the reduction of alamarBlue (Fig. 7B). We found that reduction of alamarBlue was dependent on the level of PPARγ expression. Even clone 4, which had a relatively high residual level of PPARγ expression, showed about 75% activity compared with Ski-CEFs. The observation that three different shRNAs targeting PPARγ reversed the overall metabolic activity of Ski-CEFs to that of

FIGURE 5. Ski up-regulates PPARγ and co-activates PPARγ-dependent gene transcription. A and C, expression of PPAR genes and PPARγ target genes in Ski-CEFs and CEFs measured by q-RT-PCR. Threshold cycle values given (ΔCt) were normalized to the ΔCt values of CEFs. Values given are means ± S.D. A, n = 6, ***, p < 0.001. B, CEFs, Ski-CEFs, and PPARγ-KD-Ski-CEFs were cultured in the presence of 2H2O for 24 h, and triglyceride-derived glycerol and palmitate were measured by mass spectrometry. C, newly synthesized palmitate. Values are the means ± S.D., n = 3, *, p < 0.05.
CEFs indicated that the effect was not due to an off-target knockdown. Three clones with the lowest PPARγ protein levels and the lowest alamarBlue™ reduction activity (clones 3, 9, and 10) were pooled and further analyzed. The results show that the induction of PPARγ target genes involved in lipid oxidation and transport or mitochondrial biogenesis was drastically decreased when the elevated PPARγ expression was reversed in Ski-CEFs (Fig. 7C). In PPARγKD-Ski-CEFs, expression of PPARγ target genes was decreased to about that observed in CEFs. The induction of FABP-3 and FABP-4 in PPARγ knockdown cells was not completely blocked, but compared with Ski-CEFs, it was reduced by 95 and 88%, respectively.

Analysis of respiration of both intact and permeabilized cells indicated that mitochondrial oxygen consumption by clones of PPARγKD-Ski-CEFs was reduced to the level noted in CEFs (Fig. 7, F and G). Similarly, knocking down PPARγ expression in Ski-CEFs also resulted in increased lactate production and glucose utilization, suggesting the entire shift in energy metabolism by Ski was reversed (Fig. 7, D and E).

Interestingly, mass spectrometric analysis of 2H-labeled lipids indicated that the levels of both de novo palmitate synthesis and newly made triglyceride were reduced in PPARγKD-Ski-CEFs compared with CEFs and Ski-CEFs (Fig. 6C). Thus, although lipid synthesis was not enhanced in Ski-CEFs, the constitutive activity of PPARγ in promoting lipid synthesis was likely necessary for the metabolic shift induced by Ski.

Finally, we asked whether increased PPARγ expression was necessary for the observed increase in mitochondrial mass in Ski-CEFs. Immunoblot analysis of mitochondrial proteins demonstrated that by knocking down PPARγ, the expression of proteins of the mitochondrial ETC complex, matrix, and outer membrane in Ski-CEFs was greatly reduced, although not quite to the level of CEFs (Fig. 7J). Similarly, analysis of mitochondrial mass by flow cytometry of MTG and MTR fluorescence showed that the mitochondrial mass of PPARγKD-Ski-CEFs was slightly greater than in CEFs but much less than that of Ski-CEFs (Fig. 7H). An EM of these cells demonstrated that the mitochondrial number and size of PPARγKD-Ski-CEFs was similar to that of CEFs (Fig. 7J). The combined results of these PPARγ knockdown studies establish a central role for PPARγ as a mediator of the increased fatty acid oxidation and mitochondrial mass that underlie the enhanced respiration of Ski-CEFs.

**DISCUSSION**

In this study we have delineated a molecular mechanism by which Ski shifts the energy metabolism of CEFs from glycolysis to enhanced rates of fatty acid oxidation and oxidative phosphorylation. In concert with decreased lactate production and glucose utilization (Fig. 1), our microarray analysis demonstrated that the expression of ADP-dependent glucose kinase (ADPGK) and the platelet form of phosphofructokinase in Ski-CEFs was only 25% of that noted in CEFs. Both kinases produce metabolic intermediates in glycolysis but phosphofructokinase plays a key role by catalyzing the rate-controlling reaction in the glycolytic pathway (50). Furthermore, Ski elevated the expression not only of pyruvate dehydrogenase, a subunit of the mitochondrial enzyme complex that catalyzes the conversion of pyruvate to acetyl-CoA (Fig. 4A) (51), but also of pantothenase kinase, which is a key component of the CoA synthetic pathway (52). The altered expression of these genes likely contributed to the observed increase in TCA cycle flux that accompanies Ski transformation.

The shift to oxidative metabolism in Ski-transformed cells contrasts sharply with the metabolism of fibroblasts transformed by other oncogenes, as well as most tumor cells, which have dramatically elevated aerobic glycolysis (53). This difference raised the question of whether the shift to oxidative metabolism plays an obligatory role in Ski transformation. We have found that CEFs expressing a well studied transformation-defective Ski mutant (ΔZ3/4) (4) acidify culture medium and reduce alamarBlue™ at the same rate as normal CEFs. Furthermore, microarray and q-RT-PCR results showed that this mutant does not induce any of the lipid metabolic genes that are induced by wild-type Ski, including PPARγ and PGC1-α, which control the transcription of genes involved in the control of lipid metabolism (supplemental Fig. S2). These results suggested that Ski-induced transformation and altered metabolism are highly correlated. Conversely, we have found that knockdown of PPARγ in Ski-CEFs does not reverse their phenotypic transformation. This result provided evidence against an obligatory role for enhanced oxidative metabolism in cellular transformation by Ski.

Regardless of their possible connection to transformation, our results provided insights into an unexpected functional inter-relationship between Ski and PPARγ. We found that under the influence of Ski, PPARγ mainly acts to promote oxidative metabolism in general and fatty acid β-oxidation specifically. This might be an expected role for PPARα or PPARβ, which have been shown to regulate fatty acid oxidation and catabolism (20). However, unlike PPARγ, neither of these genes was up-regulated in Ski-CEFs. Our focus on PPARγ was stimulated by the concordance of our gene expression data with published studies demonstrating that administration of the highly selective PPARγ agonist, GW1929, to rats resulted in increased expression of PPARγ target genes involved in lipid uptake and lipid oxidation in brown adipose tissue and white adipose tissue (54). Among these PPARγ target genes, CD36 and FATP-1 were shown to enhance palmitate oxidation.
through their role in delivering long chain fatty acids to carnitine palmitoyltransferase for transport into mitochondria (55–57). These two, and several additional PPARγ-regulated genes involved in the import and intracellular transport of fatty acids, were found to be up-regulated by Ski; these include LPL, FABP3, and FABP4. Through its interaction with CD36, lipoprotein lipase functions in the import of extracellular lipids into cells. Interestingly, our microarray data also showed that angiopoietin-like 4 (ANGPTL4), a known LPL inhibitor (58), was down-regulated, and the lipid carrier protein ApoA1 was dramatically up-regulated in Ski-CEFs. However, we did not detect increased expression of the PPARγ-regulated genes involved in lipogenesis. This finding is consistent with our stable isotope incorporation studies, which indicated that de novo fatty acid synthesis was decreased in response to Ski. Despite this, the level of newly synthesized triglycerides was maintained in Ski-CEFs, almost certainly a consequence of increased import of extracellular lipids.

In addition to the genes involved in lipid transport, we also detected increased expression of PPARγ-regulated genes that play supporting roles in lipid metabolism. One of them, PGC1-α, which is a transcriptional co-activator with PPARγ, is known to regulate genes that promote fatty acid oxidation, cell respiration, and nonshivering thermogenesis (26). In vivo, PGC1-α drives the formation of oxidative muscle, and PGC1-α transgenic mice display enhanced exercise ability and oxygen uptake, suggesting increased utilization of fatty acids (59). Panthothenate kinase is another PPARγ target that was found to be up-regulated by Ski. By catalyzing the first rate-limiting step in the synthesis of CoA (52), it is a likely contributor to the increased β-oxidation we observed in Ski-CEFs. Finally, we noted an up-regulation of expression of the genes for LPL (5-fold), glycerol kinase (5-fold), and glycerol-3-phosphate dehydrogenase (11-fold), in the Ski-CEFs as compared with control cells (supplemental Table S1). These enzymes form a pathway that starts with the hydrolysis of triglyceride to generate free fatty acid and glycerol and the subsequent reactions that convert the glycerol to the glycolytic intermediate dihydroxyacetone phosphate, which can then be further converted to pyruvate for subsequent oxidation via acetyl-CoA in the TCA cycle. Taken together, our results indicate that Ski forms a selective partnership with PPARγ to promote the utilization of extracellular fatty acids to produce energy via enhanced β-oxidation. Surprisingly, our results also clearly implicated PPARγ in the observed decrease in the utilization of glucose for energy production via glycolysis as this effect of Ski was reversed upon knockdown of PPARγ in Ski-CEFs.

Studies of Ski transgenic mice show that Ski regulates body composition by increasing type IIb muscle fibers in which Ski is selectively overexpressed, while decreasing adipose tissue mass (60, 61). A more recent analysis of the affected muscles in those mice demonstrated a decreased expression of several genes involved in lipid synthesis, indicating that Ski suppresses lipogenesis, consistent with our findings in Ski-CEFs (17). Those studies also agree with our results by showing that increased expression of Ski enhanced the capacity of skeletal muscle to oxidize fatty acids. Moreover, the activity of several mitochondrial enzymes was elevated in the affected muscles suggesting an increase in mitochondrial mass similar to that of Ski-CEFs. Despite the concordance of these in vitro and in vivo metabolic effects of Ski, the expression of PPARγ was not elevated in the affected muscles of the transgenic mice. The likelihood that this difference may be due to the difference between the types of cells involved is supported by studies in rats treated with the PPARγ agonist, GW1929 (54). In skeletal muscle of the treated animals, the expression of genes involved in lipid uptake and oxidation was decreased, whereas expression of genes involved in lipid synthesis was increased, although both sets of genes are known to be induced by PPARγ in other tissues. The situation was completely the opposite in adipose tissue (54). The discrepancies between observations on cultured cells and two animal models are likely the result of tissue and species specificity of the role PPARγ plays in lipid metabolism, due to differences in expression of other factors that modify or overcome PPARγ regulation.

It is also possible that Ski can produce similar changes in cellular energy metabolism by activating both PPARγ-dependent and -independent pathways. A likely candidate for a PPARγ-independent pathway is that activated by c-Myc, which has been shown to promote glutaminolysis, leading to the entry of glutamate into the TCA cycle via α-ketoglutarate (62). This is of particular significance in the present context, as we observed that oxidation of glutamate was increased in Ski-CEFs by almost 2-fold more than that of any of the other tested metabolites. In fact, our DNA microarray studies showed that Ski induced c-Myc expression by about 1.7-fold.

It has also been shown that c-Myc, like PGC1-α, acts to stimulate mitochondrial biogenesis (25, 63, 64), which was increased by Ski in CEFs. Moreover, the expression of HIF-1, which inhibits mitochondrial biogenesis and cell respiration by promoting c-Myc degradation (48), was decreased in Ski-CEFs (supplemental Table S1). The combined effect of increased c-Myc and decreased HIF-1 expression might enhance the activity of the Myc-regulated pathway to increase mitochondrial biogenesis in Ski-CEFs, independently of the PPARγ/PGC1 pathway. This speculative possibility is supported by our observation that Ski-induced mitochondrial biogenesis was not completely reversed in PPARγ knockdown cells (Fig. 7, H–J). Regardless of the possible involvement of Myc, it is clear from our knockdown results that PPARγ is the major mediator of Ski-induced mitochondrial biogenesis in CEFs. The resulting increase in mitochondrial mass provides the machinery for the shift in energy production pathways from glycolysis to β-oxidation in Ski-CEFs.

Although the emphasis in this study has been on alteration in lipid and carbohydrate metabolism, it is apparent that the metabolism of amino acids has been markedly altered in Ski-CEFs. These cells are cultured in a medium that contains essential amino acids and includes 2.5 mM glutamine, which is required for the normal growth of cells in culture. The rate of oxidation of glutamate (which is generated directly from glutamine) increased by 6.7-fold in Ski-CEFs, as compared with control cells (Fig. 2). Our data also predict an increase in the rate of synthesis of glutathione, because expression of the rate-limiting enzyme in the synthesis of glutathione, glutamate-cysteine ligase, is induced 6-fold by the introduction of Ski to CEFs.
There is also a very marked increase in the level of mRNA for the various members of the glutathione S-transferase family of enzymes in the Ski-CEFs; this group of enzymes are transmembrane proteins, which have highly divergent functions but generally catalyze the conjugation of reduced glutathione to electrophilic residues on target proteins. In addition, we noted an induction in gene expression for glutaredoxin and glutathione reductase. The data underline the key role of glutathione in a number of processes required for cell growth and metabolism, such as amino acid transport, regulation of the nitric oxide cycle, and is the major anti-oxidant that is produced by cells. It should be noted that the effects of Ski on cellular energetics reported here and in the transgenic mouse studies are in response to overexpression of Ski. The findings are therefore relevant to the role of Ski in oncogenesis, which also involves its elevated expression (6, 7, 10). Results reported in a recent publication by Elledge and co-workers (65) suggest that our findings will likely have much broader applicability. They identified Ski in a screen for genes that promote mitochondrial biogenesis in normal muscle cells. No further analysis of Ski was reported, so future work will be required to determine whether the mechanisms described here underlie the promotion of oxidative metabolism by Ski in those cells and in other cell types.

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