

Metastasis Suppressor KISS1 Seems to Reverse the Warburg Effect by Enhancing Mitochondrial Biogenesis

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Abstract

Cancer cells tend to utilize aerobic glycolysis even under normoxic conditions, commonly called the "Warburg effect." Aerobic glycolysis often directly correlates with malignancy, but its purpose, if any, in metastasis remains unclear. When wild-type KISS1 metastasis suppressor is expressed, aerobic glycolysis decreases and oxidative phosphorylation predominates. However, when KISS1 is missing the secretion signal peptide (Δ SS), invasion and metastasis are no longer suppressed and cells continue to metabolize using aerobic glycolysis. KISS1-expressing cells have 30% to 50% more mitochondrial mass than Δ SS-expressing cells, which are accompanied by correspondingly increased mitochondrial gene expression and higher expression of PGC1 α , a master coactivator that regulates mitochondrial mass and metabolism. PGC1 α -mediated downstream pathways (i.e., fatty acid synthesis and β -oxidation) are differentially regulated by KISS1, apparently reliant upon direct KISS1 interaction with NRF1, a major transcription factor involved in mitochondrial biogenesis. Since the downstream effects could be reversed using short hairpin RNA to KISS1 or PGC1 α , these data appear to directly connect changes in mitochondria mass, cellular glucose metabolism, and metastasis. *Cancer Res*; 74(3); 1–10. ©2013 AACR.

Introduction

Metabolic reprogramming of cells has long been appreciated to contribute to oncogenesis (1). First described by Otto Warburg in the 1920s, cancer cells have increased conversion of glucose to lactic acid even under normoxic conditions (2–5). As cellular metabolic signaling and primary energy sensors, mitochondrial bioenergetic and, much less commonly, genetic abnormalities mediate tumor transformation and progression (3, 6–8). Likewise, tumor-associated gene expression and/or protein activities (e.g., TP53, MYC, RAS, SRC, and HIF1 α) drive metabolic sensing (9–11), mitochondrial cristae structure (10, 12–14), as well as glucose uptake, lactate accumulation, and cytosolic pH acidification. Correspondingly, mutations in patients with cancer for the citric acid cycle enzymes (e.g., isocitrate dehydrogenase, fumarase, and succinate dehydrogenase) have been described (15) as have mutations in mitochondrial DNA (mtDNA) itself (16–18). Mutations in mitochondrial enzymes and mtDNA are relatively rare, i.e., of

insufficient frequency to explain a majority of metabolic reprogramming observed in cancers. Yet, the molecular mechanisms underlying metabolic reprogramming remain elusive and the relationship (i.e., cause–effect vs. correlation-only) to metastasis remain unclear. Two hypotheses are supported by experimental data: (i) mitochondria generate numerous reactive oxygen species (ROS), which cause oxidative stress and signal to drive cancer cell motility/invasion and tumor progression (16, 19, 20); and (ii) redox potentials or NAD⁺/NADH ratio regulate metastatic potential (16, 20–23).

Despite well-established associations of aerobic glycolysis with tumor development, the relationships, if any, with metastasis development are much less clear. Given the enormous energy requirements of the metastatic cascade, the stresses cells experience throughout the process and the flexibility of the energy metabolism by glycolysis, it makes sense that some relationship would exist (24). Recent studies indicated that specifically reduced glucose oxidation enhances tumor metastasis (25), whereas other studies failed to identify correlations (18, 26). Generation of cybrids, cells that retain their nuclear genome but have mtDNA transferred from another source, show that mitochondrial polymorphisms can dramatically influence metastatic potential (16, 18, 27). Yet, metabolic changes have not been systematically correlated with metastatic behaviors.

KISS1 is a member of the still-expanding family of metastasis suppressors, which are defined by their ability to block metastasis without preventing primary tumor development. Nascent KISS1 is a 145-amino acid polypeptide that is secreted and processed by prohormone convertases into kisspeptins (KP). KP54 [(aa68 - aa121) originally called metastatin (28) but standardized nomenclature has recently

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been adopted (29)] was first identified as the ligand for a G-protein-coupled receptor, KISS1R (also known as GPR54, AXOR12, or hOT7T175; refs. 28, 30, 31). If the secretion signal peptide is removed (designated by Δ SS), the metastasis-suppressing capacity of KISS1 is lost (32). KP54 can be further cleaved into smaller KP comprised of 13, 14, or 10 amino acid residues from the (typically amidated) C-terminal portion of KP54 (30). As long as the smaller KP retain the terminal RF-NH₂ sequence, they can bind to and activate KISS1R. KISS1/KP54 activates a variety of signals, including phospholipase C (PLC), protein kinase C, intracellular Ca²⁺ mobilization, and mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways (33). These observations led us to hypothesize that KISS1 secretion, processing, and autocrine signaling through KISS1R were responsible for antimetastatic effects. However, since none of the cell lines suppressed for metastasis when KISS1 is re-expressed express the KISS1R (32), the hypothesis was revised to implicate intracrine or paracrine signaling, or the existence of another KISS1 receptor (33).

To begin addressing these alternative hypotheses and whether there is a relationship between KISS1 metastasis suppression and metabolism, we performed bioenergetic and metabolic studies. Our results show, for the first time, that KISS1 expression increased extracellular pH by decreasing aerobic glycolysis, apparently via pathways that enhance mitochondrial respiration and/or biogenesis through regulating PPAR γ coactivator-1 (PGC1 α).

Materials and Methods

Cell lines and culture

The majority of experiments presented in this report were performed with a highly metastatic subclone of the human melanoma cell line C8161. Validation studies of key experiments were performed in MDA-MB-435 and MelJuSo. KFM (KISS1-FLAG-Metastin) or KFM Δ SS were described previously (32) in C8161.9. Briefly, KFM and KFM Δ SS insert the FLAG epitope within the proprotein convertase processing site (between R67 and X68) so that KISS1 protein and subsequent processing can be tracked. KFM Δ SS removes the 19 amino acid secretion signal sequence. Initially, constructs were made in pcDNA3.1 phagemid, but lentiviral constructs have been generated using the Life Technologies Gateway platform. Metastasis suppression was equivalent (Fig. 1A). All cells were cultured as previously described in a 1:1 mixture of Dulbecco's Modified Minimum Essential Medium and Ham's F-12 medium supplemented with 5% FBS (Atlanta Biologicals or Sigma), nonessential amino acids, and glutamine. For cells expressing exogenous cDNA or short hairpin RNA (shRNA), G418 (500 mmol/L) or puromycin (5 μ g/mL) were added. All cell lines were tested using a PCR-based assay and found to be free of *Mycoplasma spp.* contamination.

Glucose uptake and lactate production

Cells were seeded in 12-well plates at a density of 1×10^5 cells per well. Culture media was collected at 48 hours and stored at -20°C until assayed. Glucose uptake was measured using the Quantichrom Glucose Assay Kit (BioAssay Systems). Absor-

bance (630 nm) was measured using Synergy H4 Hybrid Multi-Mode Microplate Reader (Biotek) and normalized to total protein. Lactate production in the medium was detected by using Enzychrom L-Lactate Assay Kit. Results were normalized to total protein.

Real-time RT-PCR and mitochondria PCR array

To measure gene expression, total RNA was isolated and mRNA was reverse transcribed. Resulting cDNA was then amplified using TaqMan or SYBR Green probes (Primer/probe information in Supplementary Table S1). To simultaneously analyze mitochondria-associated gene expression, RNA was extracted and genomic DNA was eliminated by DNase treatment followed by PCR Array Procedure. Human mitochondria RT² profiler PCR Array and RT² Real-Timer SYBR Green/ROX PCR Mix were purchased from SuperArray Bioscience (Qia-gen). PCR was performed on ABI Prism 7700 Sequence Detector (Life Technologies).

Mitochondrial function

To measure mitochondrial function, a Seahorse Bioscience XF24 extracellular flux analyzer was used (34, 35). The rates of oxygen consumption (OCR) and extracellular acidification rate (ECAR) were expressed in pmol/min and mpH/min, respectively, and normalized by total amount of protein of cells. The mitochondrial toxins oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and antimycin A were used to disrupt mitochondrial function and were purchased from Sigma.

Antibodies, immunoprecipitation, and immunoblot

For coimmunoprecipitation experiments, cells were lysed in ice-cold lysis buffer (45 minutes, Pierce, Thermo Scientific) and centrifuged ($12,000 \times g$; 15 minutes; Beckman-Coulter Microfuge 22R Centrifuge). The supernatant was incubated with monoclonal antibody followed by precipitation with protein G-sepharose beads. The beads were precipitated by centrifugation and thoroughly washed three times. Proteins bound to beads were released and analyzed by immunoblotting. For immunoblots, cell lysates were boiled (10 minutes), resolved using SDS-PAGE using 4%–20% precast polyacrylamide gels and blotted onto nitrocellulose membranes. Antibodies were purchased from the following manufacturers and were used at the titre listed: PGC1 α (1:500; Merck), NRF1 (1:500; Santa Cruz Biotechnology), GAPDH (1:1,000; Cell Signaling Technology), and KISS1 (1:500; ref. 32). Membranes were then incubated with HRP-conjugated secondary antibodies and bands were visualized using Super-Signal West Femto Substrate (Thermo Scientific).

Yeast two-hybrid protein interactions

Full-length KISS1 was used as bait in a yeast two-hybrid screen with placenta and normal breast libraries as prey (Hybrigenics).

Invasion and migration

Invasion was measured using Matrigel-coated Transwell chambers (BD Biosciences) as previously described (32), except that tumor cells were tracked using CellTracker Green CMFDA (Life Technologies). Migration was measured using a wound healing or scratch assay as previously described (32).

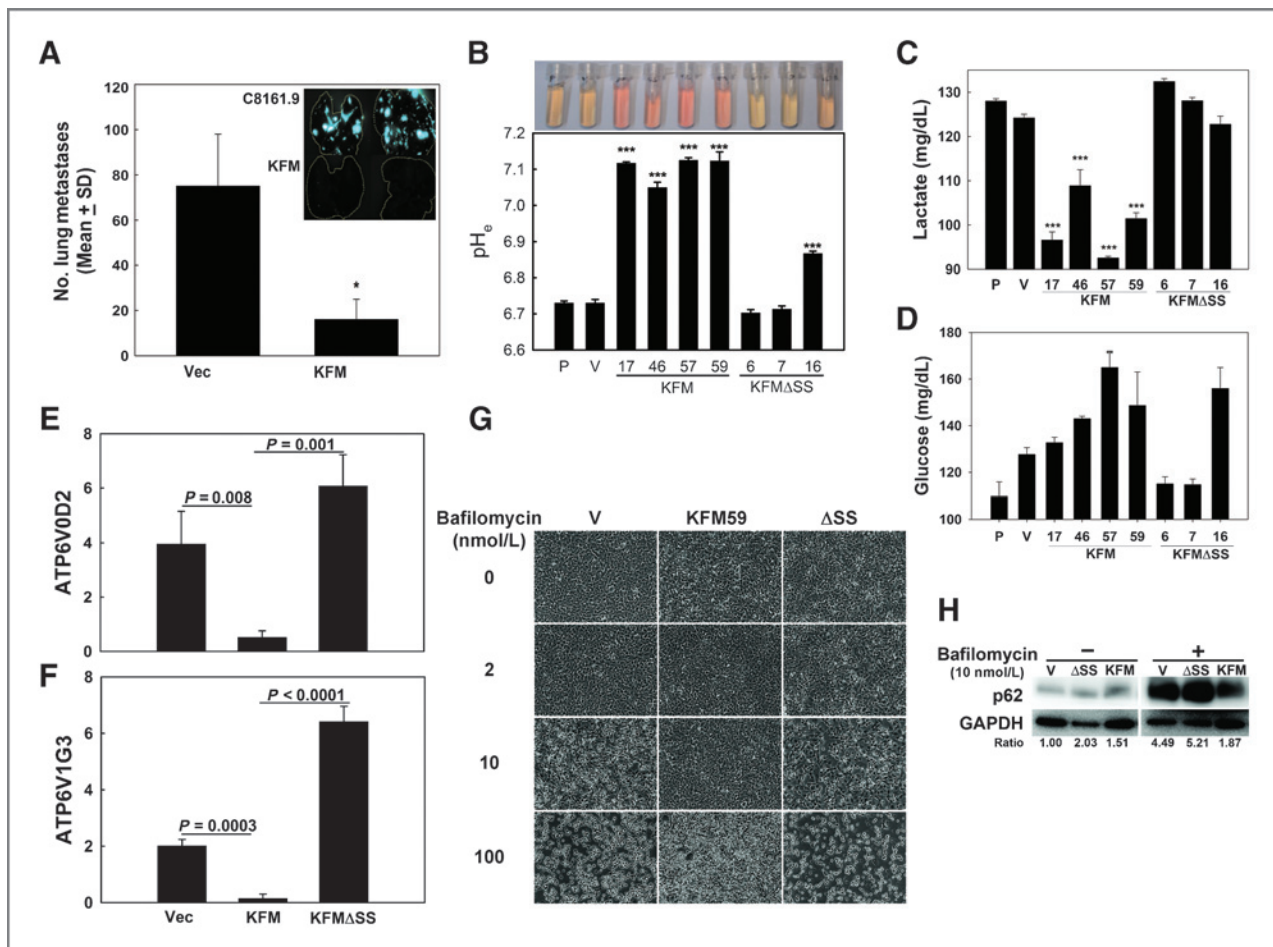


Figure 1. KISS1 downregulates extracellular acidification. **A**, KISS1 re-expression in C8161.9 cells (200,000) suppresses metastasis to lung following intravenous injection into NOD-SCID female mice ($n = 5$). Inset shows GFP-labeled tumor cells in lung 6 weeks after inoculation (*, $P < 0.05$). **B**, extracellular pH (pH_e) is significantly higher in KFM-expressing cells, but not in most KFMΔSS-expressing clones of C8161.9. Image shows cell culture medium collected from equal-density cultures, with corresponding pH measurement below. (***, $P < 0.001$, $n = 5$). **C** and **D**, lactate secretion (**C**) and glucose utilization (**D**; measured by glucose remaining in cell culture medium) by C8161.9 (P), C8161.9^{Vector} (V), C8161.9^{KFM}, and C8161.9^{ΔSS} cell clones were measured (and normalized by cell number). **E** and **F**, expression of V-ATPase V0D2 and V1G3 subunit transcripts in C8161.9^{Vector} (Vec), C8161.9^{KFM}, and C8161.9^{ΔSS} cell clones were examined by qRT-PCR. **G**, KISS1 protects C8161.9^{Vector} (V), C8161.9^{KFM}, and C8161.9^{ΔSS} cell clones from cytotoxicity following treatment with bafilomycin (0–100 nmol/L), an inhibitor of V-ATPase. **H**, immunoblot showing accumulation of p62 in bafilomycin-treated (10 nmol/L) parental and KFMΔSS-expressing, but not KFM-expressing C8161.9 cells, suggesting autophagy inhibition. Densitometry of p62 normalized to GAPDH is shown. Bars, mean ± SEM. **I**, quantification of cell viability when cells are treated with bafilomycin. Open bars, no bafilomycin; filled bar, bafilomycin-treated (10 nmol/L). ***, $P < 0.05$.

Anchorage-independent growth

Anchorage-independent growth was assessed by monitoring colony formation after 14 to 21 days in soft agar (0.8% base; 0.4% top layer) using 5,000 cells per well in 6-well plates. First, 0.6% agarose (2 mL) in growth medium was added to a 6-well plate and allowed to solidify. Then, cells were suspended in 2 mL of 0.3% agar were added on top of the agar base and allowed to solidify. Colonies (>50 cells) were stained with crystal violet and counted using a phase contrast microscope.

Statistical analyses

Experiments were done using a minimum of three replicates for each experimental group. Representative data from at least two replicate experiments are depicted. However, some experi-

ments with cell lines other than C8161.9 were performed only once to confirm critical findings. For all statistical analyses, Sigmaplot, Sigmaplot, or Prism software were used. Results are reported as mean ± SD (or mean ± SE). Statistical significance was determined by a two-sided Student *t* test or one-way ANOVA followed by Tukey post-test. Statistical significance was defined as $P < 0.05$.

Results

KISS1 expression inhibits extracellular acidification

Compared with normal tissues, the extracellular space of most tumors is acidic because production of excess lactic acid occurs during aerobic glycolysis. Growing evidence suggests that tumor acidity correlates with cancer proliferation, invasion, metastasis, and chemoresistance (36, 37). Cultured

metastatic C8161.9 cells and C8161 expressing KFMΔSS (C8161.9^{ΔSS}) cells exhibit typical acidic pH_[Ex] (range = 6.7–6.9). In contrast, medium collected from cultures of non-metastatic C8161.9 cells stably expressing KFM (C8161.9^{KFM}) maintain a neutral pH (pH_[Ex] = 7.2–7.4; Fig. 1B). Similar pH normalization following KISS1 re-expression, but not KISS1ΔSS, was observed in MDA-MB-435 and MelJuSo cell lines as well (Supplementary Figs. S1 and S6). Since production of lactate corresponds to high rates of glycolysis, lactate secretion and glucose uptake were measured (Fig. 1C and D, respectively). We further examined whether KISS1 affects enzymes in the glycolytic pathway. Transcripts of hexokinase II (HKII) were significantly decreased in C8161^{KFM} and C8161^{KFMΔSS} cells compared with vector-only cells, whereas glucose transporter GLUT1 was significantly increased. In contrast, phosphofructokinase 1 (PFK1) and LDHB genes were upregulated in C8161.9^{KFM} cells, but not C8161.9^{KFMΔSS} cells (Supplementary Fig. S1). However, expression of glycolytic enzymes were not consistently observed in MelJuSo melanoma cells. Therefore, KISS1-mediated metabolic changes should be ascribed elsewhere. KISS1-expressing cell clones showed lower glucose uptake and lactate secretion.

Lactate is not the sole contributor to acidic microenvironments (37, 38). Plasma membrane vacuolar proton-ATPase (V-ATPase) also promotes extracellular acidification as well as increased invasion, survival, and metastasis (39–41). A comprehensive analysis of v-ATPase subunit expression by qRT-PCR revealed that expression of the V0d2 and V1g3 isoform transcripts were significantly decreased in C8161.9^{KFM} cells compared with parental of KFMΔSS-expressing tumor cells (C8161.9 or C8161.9^{ΔSS} shown in Fig. 1E and F, respectively). If conditioned medium from KFM, but not parental or KFMΔSS cell cultures, were put onto vector-only transfected C8161.9 cells, both V-ATPase transcripts were also reduced (Supplementary Fig. S2A). Bafilomycin, an inhibitor of v-ATPase, induced cell death in C8161.9 and C8161.9^{ΔSS} cells, whereas C8161.9^{KFM} cells were resistant at equivalent doses (Fig. 1G–I). Because bafilomycin is also an inhibitor of autophagy, we speculated that C8161.9^{KFM} cells should be resistant to bafilomycin-induced autophagy inhibition (i.e., undergo apoptosis in response to stress). KISS1 showed substantially lower p62 accumulation (i.e., deficient for autophagy; Fig. 1H). Moreover, significant upregulation of apoptotic genes (BNIP3 and ATG6) and glycolytic pathway factors (GLUT1, HKII, PFK1) were observed in vector- and KFMΔSS-, but not KFM-expressing cells (Supplementary Fig. S2B). Taken together, these data strongly indicate that KISS1-expressing cells are less V-ATPase active and, therefore, may have a deficiency in autophagy compared with vector- and KFMΔSS-expressing cells.

KISS1 induces mitochondrial biogenesis and activation

Reduced acidification in KISS1-expressing cells suggested that they might have shifted from glycolysis to oxidative phosphorylation. A Seahorse Bioscience XF24 bioanalyzer was used to examine multiple mitochondrial function parameters after sequential injection of oligomycin (ATP synthase

inhibitor), FCCP (uncoupling agent), and Antimycin A (Complex III inhibitor; Fig. 2A and B; ref. 35). These parameters include OCR, ATP-linked respiration, and the reserve respiratory capacity (Fig. 2A and B).

Consistent with lactate measurements, ECAR (Fig. 2C) was significantly lower in C8161.9^{KFM} cells compared with C8161.9^{vector} and C8161.9^{ΔSS}, whereas C8161.9^{KFM} cells exhibited significantly higher basal oxygen consumption, ATP-linked respiration, and reserve capacity (Fig. 2D–F). Together, these results confirmed that KISS1-expressing cells shifted from glycolysis to oxidative phosphorylation and enhanced bioenergetic capacity. These changes may have resulted from either improved functionality of existing mitochondria or enhanced mitochondrial function associated with increased total mitochondrial mass in the KISS1-expressing cells (i.e., the per mitochondrion functionality is the same, but total oxidative phosphorylation per cell would be higher).

To assess a change in mitochondrial mass, C8161.9 and MDA-MB-435 cells were stained with MitoTracker dyes and quantified by immunofluorescence (Fig. 3A and B) and flow cytometry (Fig. 3B insets). KISS1-expressing cells consistently had significantly greater mitochondrial mass than non-expressing or KFMΔSS-expressing cells. Consistent with the MitoTracker staining results, expression of genes related to mitochondrial biogenesis and function, measured using a mitochondrial PCR array (Supplementary Fig. S3), were mostly higher (Fig. 3C). In KISS1-expressing cells, various mitochondrial genes encoded by the nuclear genome [apoptotic protein (*AIFM2*), chaperone proteins (*HSP60* and *HSP90AA1*), membrane polarization and potential proteins (*Ucp4* and *Ucp5*), import proteins (*TIMM8A*, *TIMM8B*, *IMMP1L*, *MIPEP*, and *LRPPRC*), and small molecule transport and import/fission proteins (*TSPO*, *SLC25A12*, *SLC25A20*, *SLC25A23*, *MFRN*, *ANT1*, and *ANT2*)] were consistently more highly expressed. Furthermore, re-expression of KISS1 markedly induced expression of two key transcription factors in C8161 and MelJuSo cells, mitochondrial nuclear respiratory factors (NRF1) and mitochondrial transcription factor A (Tfam), corresponding to higher expression of mitochondrion genome-encoded genes (Fig. 3C). Collectively, these data suggest that KISS1 promotes a coordinate metabolic shift, stimulation of mitochondrial biogenesis, and increased mitochondrial function.

KISS1 promotes PGC1α expression and differentially regulates downstream metabolism factors

Peroxisome proliferator-activated receptor-γ co-activator 1-α (PGC1α) is a transcriptional coactivator for many of the genes responsible for the regulation of mitochondrial biogenesis and function (42). PGC1α interacts with NRF1 and activates transcription of Tfam, which then activates transcription and replication of the mitochondrial genome (43). Therefore, we explored whether PGC1α is involved in KISS1-mediated metabolic changes. KISS1 did not affect *PGC1α* mRNA expression (Fig. 4A), but greatly increased steady-state PGC1α protein expression (Fig. 4B). Introduction of shRNA for KISS1 attenuated PGC1α protein expression in nuclei (Fig. 4C, left), further leading to the conclusion that there is a direct link between KISS1 and PGC1α expression.

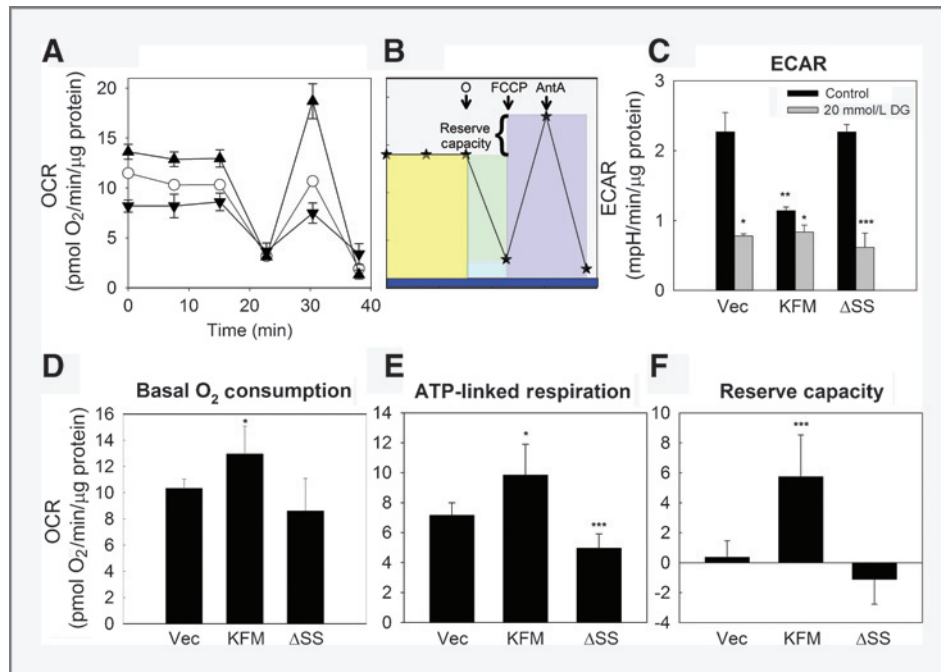


Figure 2. KISS1 enhances mitochondria respiratory capabilities. Measurement of mitochondrial function in C8161.9^{Vector}, C8161.9^{KFM}, and C8161.9^{ΔSS} cells using the XF24 bioanalyzer. A, measurement of OCR under the basal conditions followed by the sequential addition of oligomycin (O), FCCP, and antimycin A as depicted schematically in B. ○ vector; ▲, KFM; ▼, KFMΔSS. Colored blocks in B represent basal oxygen consumption (yellow); ATP-linked respiration (green); maximal oxygen consumption (purple); proton leak (light blue); and other respiration (dark blue). Reserve capacity is the difference between maximal ATP-linked respiration and maximal oxygen consumption. C, ECAR measured contemporaneously with OCR measurements in the absence (black bars) or presence (gray bars) of 2-deoxyglucose (2-DG; 20 mmol/L). D, basal oxygen consumption. E, ATP-linked respiration. F, reserve capacity derived from OCR measurements in A. N = 6–8; error bars, SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To assess whether KISS1 affects stability of PGC1 α protein, cells were treated with cycloheximide to prevent protein synthesis. Figure 4C (right) shows that PGC1 α levels quickly dropped in C8161.9 and C8161.9^{ΔSS}, but no reduction was observed in C8161.9^{KFM} cells, suggesting that KISS1 somehow stabilized PGC1 α protein expression at a posttranslational level.

Since PGC1 α regulates multiple aspects of energy metabolism including mitochondria biogenesis, fatty acid oxidation, fatty acid synthesis, glucose utilization, and antioxidant detoxification, KISS1 alterations of PGC1 α downstream signals was measured (Supplementary Fig. S4). KISS1 downregulated expression of PPAR α (fatty-acid oxidation), but upregulated ACC and FASN (fatty acid synthesis). Knockdown of KISS1 using shRNA blocked KISS1 induced changes of these genes. These intriguing results suggest that KISS1 differentially (i.e., not ubiquitously) regulates cellular metabolism through its regulation of PGC1 α .

In initially unrelated experiments searching for KISS1-interacting proteins, one of the highest probability interacting proteins (i.e., relative binding strength and frequency of interaction in breast and placental libraries) was a molecular chaperone protein, ubiquitin-like protein (PLIC-1 or Ubiquilin-1). Ubiquilin-1 reportedly binds ubiquitylated proteins and ubiquitin ligases to interfere with the process of proteasome-dependent degradation (44). Figure 4C (right) shows that Ubiquilin-1 expression was as stable as

PGC1 α in C8161.9^{KFM} cells, raising the possibility that KISS1 protects PGC1 α from degradation by interacting with Ubiquilin-1.

NRF1 was identified in the yeast two-hybrid screens as another strongly interacting KISS1-interacting protein. Using Myc-tagged NRF1, the interaction was validated using anti-Myc antibodies in transiently transfected MelJuSo cells coexpressing KISS1 (Fig. 4D). In a separate experiment, stably expressed V5-NRF1 in C8161.9^{KFM} and C8161.9^{ΔSS} cells was coimmunoprecipitated (Fig. 4E). Although C8161.9^{ΔSS} shows some interaction with V5-NRF, much more NRF1 associated with KISS1 in C8161.9^{KFM} cells.

PGC1 α is essential for KISS1-mediated metabolism changes and suppression of invasion

To investigate whether higher expression of PGC1 α in KISS1-expressing cells was relevant to KISS1-mediated metabolism changes and/or metastasis suppression, shRNA was used to diminish PGC1 α expression in C8161.9^{KFM} cells. As predicted, knockdown of PGC1 α resulted in extracellular acidification, increased glucose uptake and enhanced lactate secretion in cultured C8161.9^{KFM} cells (Fig. 5 and Supplementary Fig. S7A). mtTFA and other mitochondrial genes were concomitantly downregulated in C8161.9^{KFM} cells after knocking down PGC1 α expression (Supplementary Fig. S5).

The *in vitro* metabolic associations prompted examination of whether PGC1 α is also involved as a downstream signal of

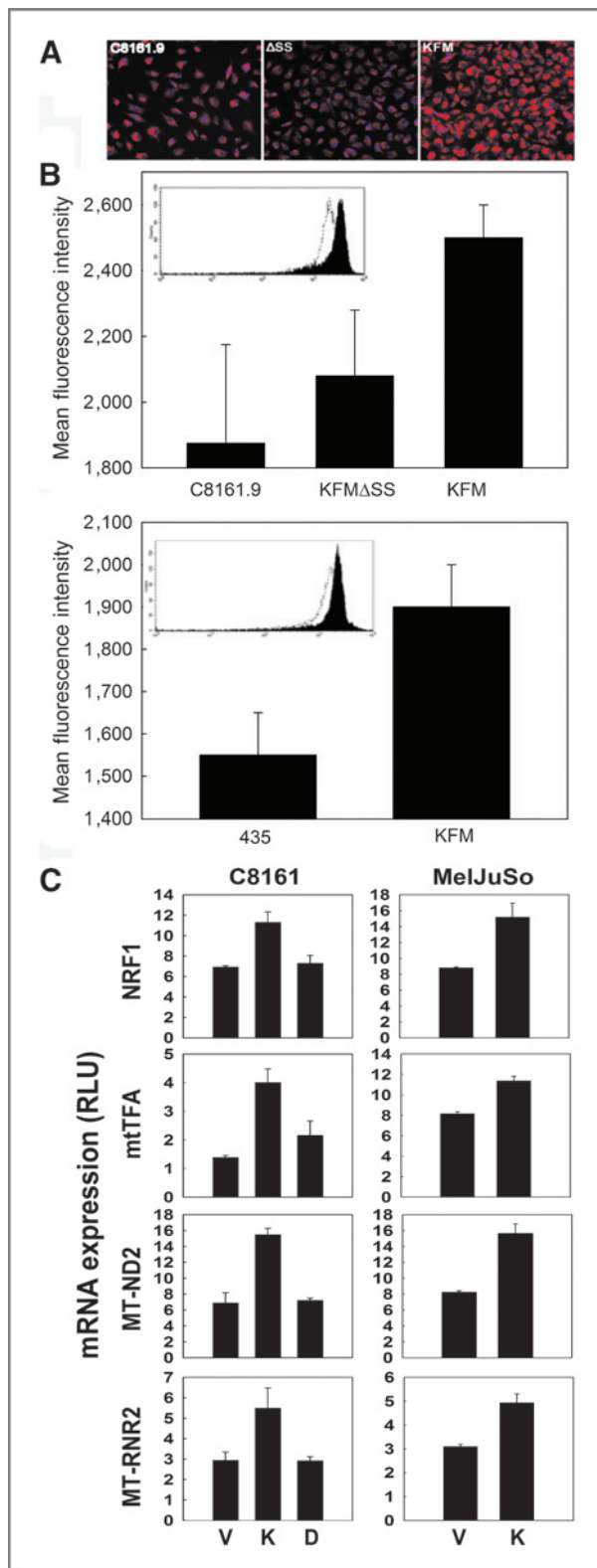


Figure 3. KISS1 enhances mitochondrial biogenesis. A, MitoTracker staining of C8161 and MDA-MB-435 cancer cells shows that KISS1- (KFM), but not KFM Δ SS-expressing cell clones, have higher mitochondrial mass than parental cells, which was validated and further

quantified by flow cytometry (B). Actual flow cytometric plots are shown in the insets. C, KISS1, but not KFM Δ SS, expression increases transcript levels of mitochondrial biogenesis-associated genes (NRF1, mtTFA) and mitochondrial genes (MT-ND2, MT-RNR2) in C8161 and MelJuSo cell lines. V, C8161.9^{Vector}; K, C8161.9^{KFM}; D, C8161.9^{KFM Δ SS}. Bars, mean \pm SEM, $n = 3-5$.

Discussion

The glycolytic phenotype that persists in most primary and some metastatic cancers, even during normoxic conditions, would appear to provide a strong selective growth advantage. Despite many hypotheses to explain cancer cell predilection toward aerobic glycolysis (6, 45), the underlying mechanisms are still being uncovered as debates concerning the selective advantages of the Warburg effect continue (46–48). We report here that the KISS1 metastasis suppressor inhibits aerobic glycolysis and increases oxidative phosphorylation, strongly suggesting that aerobic glycolysis is not required for primary tumor growth, but that it may contribute to successful metastasis.

The effects of KISS1 on glucose metabolism and microenvironment acidification provide plausible explanations for differences in metastasis between cell clones in a tumor. Acidosis can be mutagenic as it can inhibit DNA repair (49), which, in turn, could promote mutations that lead to metastatic competency. Lowering extracellular pH can impede cell-cell communication through gap-junctions (50), possibly altering cellular reception of growth regulatory signals. Extracellular pH also regulates activation, secretion, and cellular distribution of many proteases (51–53), some of which are involved in breakdown of the extracellular matrix and invasion. All of these consequences of metabolic shifts could affect metastasis development.

Beyond enhanced glycolysis, there are additional mechanisms that can lead to extracellular acidification. Proton pumps, such as the vacuolar H⁺-ATPases (v-ATPase), which are ubiquitous multi-subunit ATP-dependent proton pumps found within plasma membrane, endosomal, lysosomal, and Golgi-derived cellular membranes (54–56), contribute to membrane potentials and microenvironment pH. Plasma membrane-associated v-ATPase has been implicated in metastatic tumor cells (39–41). In addition to the metabolic changes occurring when KISS1 is re-expressed, we found that KISS1 appears to regulate v-ATPase expression, leading to the notion that manipulation of the microenvironment might be an underlying mechanism by which KISS1 allows growth at orthotopic sites while not allowing it at ectopic (metastatic) sites.

The most surprising and most profound observations reported here relate to KISS1 expression boosting mitochondrial biogenesis via modulation of PGC1 α expression. Importantly, PGC1 α seems to be essential for KISS1-mediated

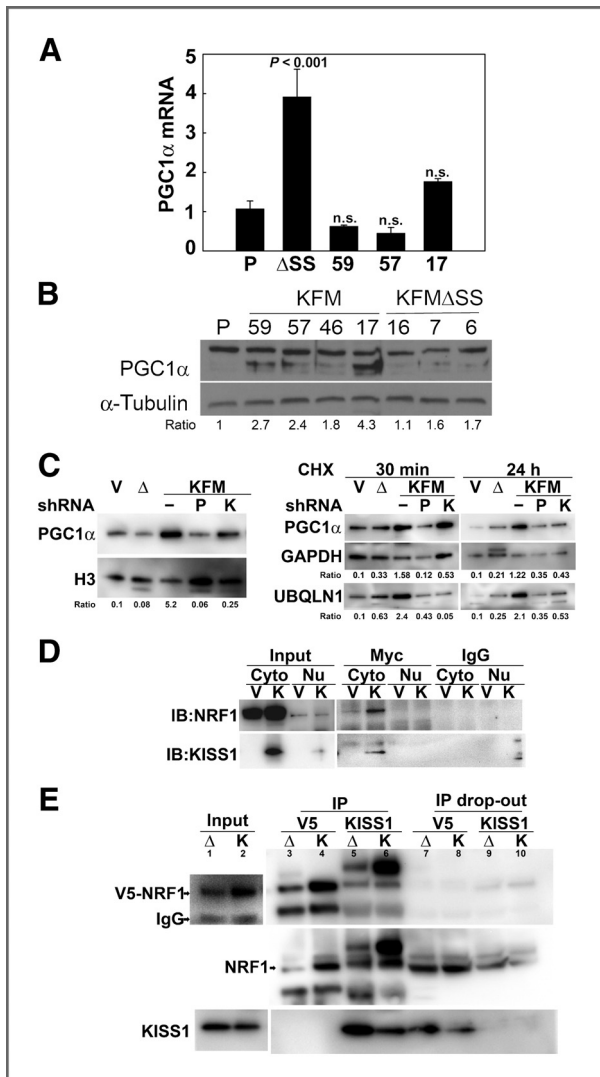


Figure 4. KISS1 upregulates PGC1α expression and differentially regulates PGC1α downstream signals. **A**, KISS1 does not increase PGC1α mRNA as detected by qRT-PCR; however, PGC1α protein (**B**, immunoblot, lower band) is consistently higher in KISS1-expressing, but not KFMΔSS-expressing C8161-derived cell clones. **C**, treatment of KISS1-expressing (KFM) cells with shRNA to KISS1 reduces PGC1α in the nucleus of C8161 cell clones (left). Right, C8161.9^{Vector} (V), C8161.9^{ΔSS} (Δ), C8161.9^{KFM} (K), C8161.9^{KFM/shKiss1} (K), and C8161.9^{KFM/shPGC1α} (P) cells were treated with cycloheximide for 30 minutes and 24 hours followed by immunoblotting to examine the expression of PGC1α and ubiquitin 1 (Ubql1). **D**, MeJuSo (V) and MeJuSo (K) cells were transfected with Myc-tagged NRF1 plasmid. Cells were lysed and immunoprecipitated using anti-Myc antibody or control IgG, followed by immunoblotting with anti-NRF1 or anti-KISS1 antibodies. Data show that KISS1 interacts with NRF1 in the cytoplasm and is validated in reciprocal immunoprecipitation studies. **E**, following transduction with V5-tagged NRF1, cells were lysed and immunoprecipitated using anti-V5 or anti-KISS1 antibodies followed by immunoblotting with anti-NRF1 or anti-KISS1 antibodies. Data show that KISS1 interacts with NRF1, but KFMΔSS, does not.

metabolic changes and invasion/metastasis suppression; that is, there appears to be a KISS1-PGC1α pathway involved in controlling malignant behavior. Roles for PGC1α in cancer are

not unprecedented. Consistent with our findings, recent studies show that PGC1α expression is reduced in tumors compared with matching normal tissues (57–61). Moreover, PGC1α expression is inversely correlated with survival in breast cancer (62).

That PGC1α interacts with so many members of the nuclear receptor superfamily of orphan and ligand-activated transcription factors belies its versatility in controlling diverse biologic programs involved in metabolism. One of the primary functions of PGC1α is to regulate energy metabolism by increasing oxidative metabolism, particularly mitochondrial oxidative phosphorylation by inducing expression of most genes in the citric acid cycle and electron transport chain

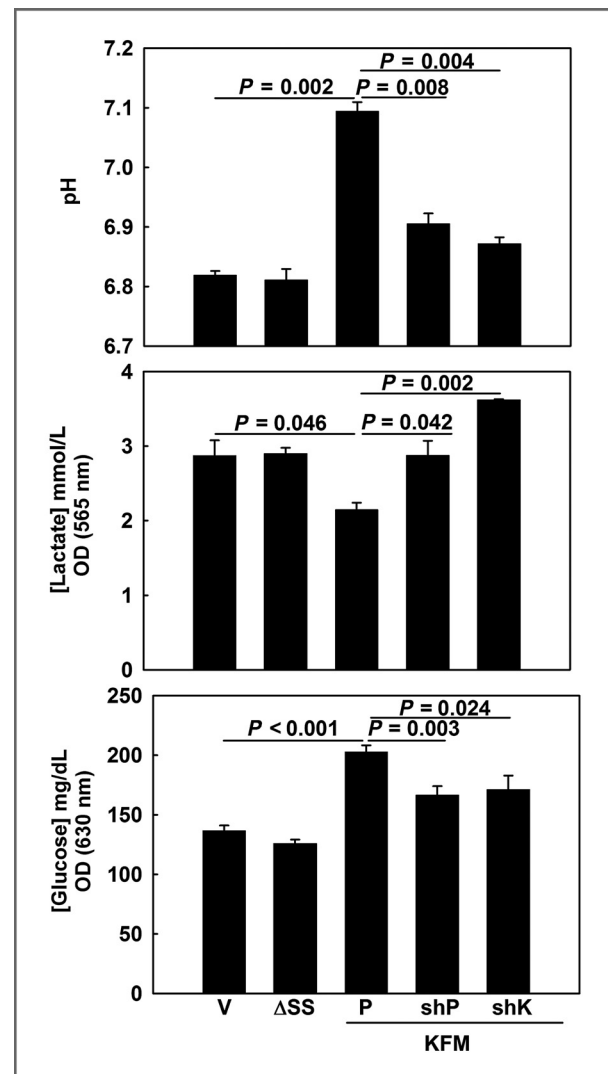


Figure 5. PGC1α is essential for KISS1-mediated metabolism changes. KISS1 effects on pH, lactic acid production, and glucose uptake are reversed when KISS1 or PGC1α are knocked down using shRNA (shK and shP, respectively). Representative data are shown, but data are equivalent using other shRNA from Open Biosystems. Bars, mean \pm SEM; $n = 3$. P values calculated using one-way ANOVA followed by Tukey post-test.

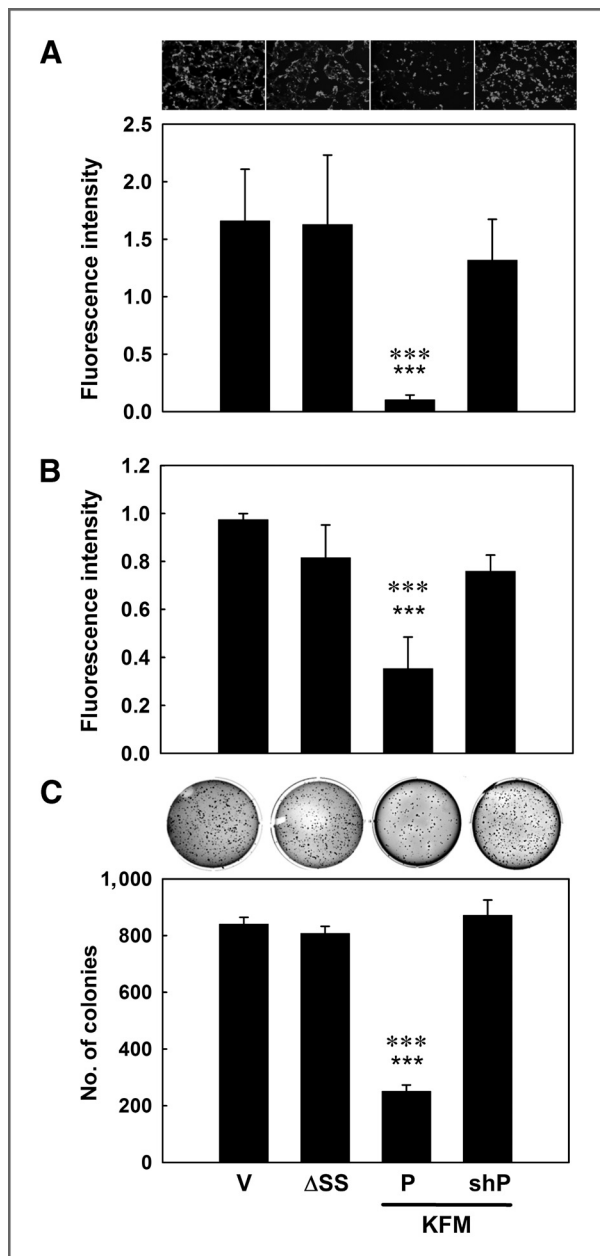


Figure 6. PGC1 α is essential for KISS1 metastasis suppression. A, C8161.9^{Vector} (V), C8161.9 Δ SS C8161.9^{KFM} (P), and C8161.9^{KFM/shPGC1 α} [KFM- (SHK) and shRNA to PGC1 α (SHP) cotransfected] cell clones were evaluated for Transwell invasion (A) and migration using a scratch assay (B) and anchorage-independent growth in soft agar (C). Representative images for invading cells and colonies in soft agar are shown. Bars, mean \pm SEM, $n = 3$; three stars, $P < 0.001$ compared with vector or KFM Δ SS; ***, $P < 0.001$ compared with shRNA to PGC1 α (shP).

(43). Mitochondrial biogenesis and respiration are, as a result, stimulated by PGC1 α through powerful induction of the transcription factors NRFs and mtTFA (63). Thus, the mitochondrial biogenesis associated with KISS1 expression is probably through upregulation of NRF1 and mtTFA.

PGC1 α also regulates anabolic metabolism, which addresses one of the presumed benefits of cellular adoption of aerobic glycolysis—a shift toward macromolecular synthesis rather than efficiency of nutrient-to-energy conversion. For example, under nutrient-rich conditions, PGC1 α promotes *de novo* fatty acid synthesis by coactivating the lipogenic transcription factor SREBP1 (64, 65). Under conditions of nutrient deprivation, PGC1 α promotes β -oxidation by coactivating the liver X receptor (LXR; refs. 64, 66, 67). When KISS1 is re-expressed, PGC1 α downstream genes involved in β -oxidation (PPAR α) are downregulated while genes involved in lipogenesis (FASN, ACC1) are upregulated. KISS1 re-expression also inhibits activation of AMPK (Supplementary Fig. S7B), a major regulator in cellular energy homeostasis and autophagy. In highly proliferating metastatic cancers (of which all of the parental cell lines used in this report represent), cells at the core of the tumor have limited access to ATP and oxygen, which are essential for growth and survival. These conditions would lead to upregulation of glycolysis, inhibition of mitochondrial ATP synthesis, and AMPK activation that, in turn, inactivates ACC1/2 to maintain NADPH levels. β -oxidation serves as an alternative survival pathway and several reports show that inhibition of mitochondrial β -Oxidation compromises tumor cell survival (68–71). Collectively, these changes would enable cells to generate significant amounts of energy and promote cell survival during energy stress conditions.

On the other hand, we found that KISS1 cells have decreased V-ATPase expression. Preliminary studies suggest that KISS1 may prevent the assembly of V0 and V1 domains (data not shown). As a result, KISS1 cells have massively attenuated v-ATPase activity (which is required for the final step of autophagy, the breakdown of cargo delivered to the vacuole). Reduced autophagy in KISS1 cells was confirmed by increased p62 protein. Recent work has shown that p62 controls cell survival, autophagy, and apoptosis. Moreover, modulation of p62 by autophagy is a key factor in tumorigenesis. Although these data suggest a connection between KISS1, metabolic regulation via autophagy, apoptosis, and survival, more extensive studies are needed to firm up the associations.

Our findings establish a novel and unanticipated connection between the metastasis suppressor KISS1 and tumor metabolism. KISS1 regulation is multifaceted, affecting glycolysis, mitochondrial biogenesis, and lipid homeostasis. These metabolic changes appear to center around KISS1 regulation of PGC1 α protein levels, which begins to explain the paradoxical observation that the metabolism effects are observed in cells that do not express the KISS1 receptor (32) and that KISS1 mutants that are not secreted do not elicit a metabolic shift. Hints regarding a molecular mechanism of action are found in the direct binding of KISS1 and ubiquitin-1 and NRF1. Interactions between KISS1 and ubiquitin suggest that the presence of KISS1 reduces PGC1 α protein degradation that, in turn, results in overall higher PGC1 α expression. Thus far, however, we have not been able to identify a differential interaction with the KFM Δ SS mutant that would fully explain the results. On the other hand, wild-type KISS1, but not the Δ SS mutant, interacts with NRF1. This is consistent with the observation that

enhanced mitochondria biogenesis was only found in wild-type KISS1-expressing cells.

Ultimately, the findings suggest that some therapies that normalize metabolism may be particularly beneficial in controlling metastasis. Of course, this remains to be seen. This report establishes, for the first, time a direct relationship between metastasis suppression and metabolism. Emerging data using other models find similar trends, even though the mechanisms leading to normalized metabolism and metastasis suppression are not directly overlapping. Given the cross-talk between proliferation, survival and metabolic pathways and the perturbations that have been previously described in each of those pathways as relating to malignant behavior compel a more systematic and comprehensive examination of the interrelationships, particularly in the context of metastasis control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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