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Acquired resistance to metformin in breast cancer cells triggers transcriptome reprogramming toward a degradome-related metastatic stem-like profile

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Therapeutic interventions based on metabolic inhibitor-based therapies are expected to be less prone to acquired resistance. However, there has not been any study assessing the possibility that the targeting of the tumor cell metabolism may result in unforeseeable resistance. We recently established a pre-clinical model of estrogen-dependent MCF-7 breast cancer cells that were chronically adapted to grow (> 10 months) in the presence of graded, millimolar concentrations of the anti-diabetic biguanide metformin, an AMPK agonist/mTOR inhibitor that has been evaluated in multiple in vitro and in vivo cancer studies and is now being tested in clinical trials. To assess what impact the phenomenon of resistance might have on the metformin-like "dirty" drugs that are able to simultaneously hit several metabolic pathways, we employed the ingenuity pathway analysis (IPA) software to functionally interpret the data from Agilent whole-human genome arrays in the context of biological processes, networks, and pathways. Our findings establish, for the first time, that a "global" targeting of metabolic reprogramming using metformin certainly imposes a great selective pressure for the emergence of new breast cancer cellular states. Intriguingly, acquired resistance to metformin appears to trigger a transcriptome reprogramming toward a metastatic stem-like profile, as many genes encoding the components of the degradome (*KLK11*, *CTSF*, *FREM1*, *BACE-2*, *CASP*, *TMPRSS4*, *MMP16*, *HTRA1*), cancer cell migration and invasion factors (*TP63*, *WISP2*, *GAS3*, *DKK1*, *BCAR3*, *PABPC1*, *MUC1*, *SPARCL1*, *SEMA3B*, *SEMA6A*), stem cell markers (*DCLK1*, *FAK*), and key pro-metastatic lipases (*MAGL* and *Cpla2*) were included in the signature. Because this convergent activation of pathways underlying tumor microenvironment interactions occurred in low-proliferative cancer cells exhibiting a notable downregulation of the G_2/M DNA damage checkpoint regulators that maintain genome stability (*CCNB1*, *CCNB2*, *CDC20*, *CDC25C*, *AURKA*, *AURKB*, *BUB1*, *CENP-A*, *CENP-M*) and pro-autophagic features (i.e., *TRAIL* upregulation and *BCL-2* downregulation), it appears that the unique mechanism of acquired resistance to metformin has opposing roles in growth and metastatic dissemination. While refractoriness to metformin limits breast cancer cell growth, likely due to aberrant mitotic/cytokinetic machinery and accelerated autophagy, it notably increases the potential of metastatic dissemination by amplifying the number of pro-migratory and stemness inputs via the activation of a significant number of proteases and EMT regulators. Future studies should elucidate whether our findings using supra-physiological concentrations of metformin mechanistically mimic the ultimate processes that could paradoxically occur in a polyploid, senescent-autophagic scenario triggered by the chronic metabolic stresses that occur during cancer development and after treatment with cancer drugs.

In the era of personalized medicine, all initially successful molecularly targeted therapies are limited by the invariable and often rapid occurrence of resistance in tumor cells. Although new cancer drugs have been developed to specifically and efficiently interfere with defined genetic aberrations, resistance commonly occurs through the acquisition of compensatory mechanisms that bypass the function of the cancer gene that is pharmacologically targeted.[1] Interestingly, one of the available pathways that can bypass the driver status of the genetic target is a common feature across multiple types of cancer: deregulated cellular metabolism.[2-6] The metabolic properties of cancer cells are remarkably different from those of normal cells, and mounting evidence supports the idea that metabolic reprogramming is linked not only to the efficacy of classical therapeutic approaches in cancer, such as

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radiotherapy, hormonotherapy, and chemotherapy but also to the efficacy of newly developed molecularly targeted drugs.[7-19]

While it might appear intuitive that deregulated cancer metabolism can activate pro-survival signaling and decrease drug-induced apoptosis to provide a general, unspecific protection against cell injuries induced by multiple types of cytotoxicities, it is worth noting that resistance to oncogene-mediated targeted therapy has been shown to require a shift toward the very same metabolic state that is controlled by growth factor signaling.[20,21] In cancer cells sensitive to lapatinib, the small-molecule dual inhibitor of the oncogenes EGFR and HER2, receptor tyrosine kinase signaling is disrupted, and activity of its Ras, PI3K, and mTOR downstream effectors is abrogated; because oncogene-dependent metabolic rewiring is prevented, cancer cell death is observed. In drug-resistant cells, however, the resistance mechanism does not involve the expected reactivation of the Ras, PI3K or mTOR pathways, but rather involves the reactivation of multiple metabolic processes, including the unfolded protein response, autophagy, glycolysis, and gluconeogenesis, which ensures a metabolic rewiring that permits cancer cell proliferation even upon the removal of any activity from canonical growth factor signaling pathways.

The latter observations strongly confirm that mutations that activate oncogenes or inactivate tumor suppressors appear to "softwire" cancer genes to metabolism, because these cancer driver genes directly regulate metabolic enzymes.[22-25] Importantly, because metabolic reprogramming is a central (re) wiring or convergence point of many, if not all, cancer-related signaling pathways, tumor cells might be unable to adapt to the molecular challenges imposed by multifaceted drugs that act on cell metabolism at multiple levels. Not surprisingly, the area of cancer metabolism research is undergoing an unstoppable renaissance, because therapeutic interventions based on metabolic inhibitor-based therapies should be less prone to acquired resistance, assuming that the changes in tumor metabolism are similar across multiple cancerous tissues and affect many cancer cell types, including cancer stem cells (CSCs). In this regard, there have been no studies assessing the possibility that targeting tumor cell metabolism may face yet-to-be discovered resistance.

Metabolic reprogramming may not be a "passenger" phenomenon, but rather an active driver of the transformed phenotype. For this reason, we recently speculated that currently proposed antitumor drugs that target various metabolic pathways would impose great selective pressure for the emergence of resistant cells. An ever-growing amount of in vitro studies have confirmed that the anti-diabetic drug metformin can exert anticancer activity by decreasing the activation of the mammalian target of rapamycin (mTOR), a unique sensor that coordinates nutrient availability and energy metabolism with cell responses to growth factors. In vivo studies have shown that metformin can negatively affect the growth of human tumors even in the presence of activating mutations in the *PIK3CA* oncogene, another evolutionary conserved regulator of cell metabolism that converges with and impinges on the mTOR pathway.[10,26-37] To anticipate the potential mechanisms of acquired resistance to metformin during the course of treatment, we recently established metformin-resistant pooled cell populations from the MCF-7 breast carcinoma cell line. Thus, to assess what impact the resistance phenomenon might have on metformin-based therapies, genome-wide analyses using Agilent 44K Whole Human Genome Arrays were evaluated using a bioinformatics approach with the ingenuity pathway analysis (IPA) software. Here, we reveal for the first time that the genomic spaces related to chronic adaptation to the AMPK agonist/mTOR inhibitor metformin involve a degradome-related metastasis aggressiveness gene expression-like signature.

Results

To anticipate the potential mechanisms of acquired resistance to metformin during the course of treatment, we established a pooled population of metformin-adapted cancer cells from metformin-naïve MCF-7 breast cancer cells. To simulate the clinic where patients receive metformin on a daily chronic basis, we developed a model of acquired adaptation to metformin by chronically exposing MCF-7 cells to graded concentrations of metformin for longer than 10 mo before starting any experimental procedure (**Fig. 1**, left panels). We have now isolated the metformin-refractory pooled populations of MCF-7/MET-R cells that are capable of growing in the presence of 30 to 40 mmol/L metformin, a range of metformin concentrations that are highly cytotoxic to the parental MCF-7 cells, as confirmed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT]-based metabolic assays (**Fig. 1**, right panel).

Characterization of a pathway-based transcriptomic signature in MCF-7 breast cancer cells with acquired resistance to metformin

To determine the gene expression effects related to metformin efficacy in breast cancer cells, we performed genome-wide analyses by comparing the global transcriptomic profiles of metformin-naïve MCF-7 cells to those obtained from a pooled population of metformin-adapted MCF7/MET-R cells. After RNA hybridization to an Agilent 44K (double density) Whole Human Genome Oligo Microarray, which contains 45 220 probes representing 41 000 unique human genes and transcripts, the normalized and filtered data from all experimental groups were simultaneously analyzed using the SAM algorithm. Using a 2.0-fold-change cut-off value relative to the transcriptome of metformin-naïve MCF-7 parental cells, genes that showed significant expression changes were identified. Only genes with well-annotated transcripts (i.e., not partial *cds* for hypothetical proteins, hypothetical insert cDNA clones, etc.) were selected, and genes that could not be identified were eliminated. We identified 840 genes (474 upregulated and 366 downregulated) that were differentially expressed in the MCF-7/MET-R cells. **Tables S1 and S2** summarize the upregulated and downregulated gene transcripts, respectively, in the "metformin adaptation" transcriptomic signature.

To identify functions that were significantly altered under the metabolic selective pressure (i.e., metformin treatment), we used an experimental approach that focused on gene pathways. Although several computational methods have been proposed

for incorporating biological pathway information and gene sets into microarray data analysis, we decided to employ Ingenuity Pathway Analysis (IPA) using the Ingenuity® software. We utilized the "core analysis" function included in the software package to interpret the metformin resistance-related global transcriptomic profiles in the context of biological processes, networks, and pathways. The IPA software algorithmically generates networks of up- and downregulated functionally related annotated genes based on their connectivity and assigns a score that considers both the number of the focus genes in a network and the size of the network to approximate the relevance of each network in relation to the original list of focus genes. **Figure 2** graphically illustrates the 5 gene network functions that were most significantly (score > 30) upregulated (red), and **Figure 3** illustrates the 5 gene network functions that were most significantly (score > 30) downregulated (green) in the metformin resistance-related transcriptomic signature of MCF-7 breast cancer cells.

The top functions of the upregulated gene networks (**Fig. 2**) were related to: (1) Connective tissue disorders, dermatological diseases and conditions, developmental disorder (score = 41), including the cancer stem cell marker *DCLK1*, the enhancer of the cell motility and metastasis *BCAR3* (breast cancer antiestrogen resistance 3) gene, and the *PABPC1* (poly A binding protein 1) gene, a component of the ezrin-driven metastatic phenotype. Intriguingly, this gene network included the *LAMA3*, *LAMB3*, and *LAMC2* genes, which encode 3 polypeptide chains, alpha3, β3, and gamma2, respectively, of laminin 5, which anchors epithelial cells to the underlying basement membrane and negatively regulates tumor invasion, and the tumor suppressor *DACH1*, whose expression is lost in some forms of metastastic cancer but is highly expressed in other metastastic carcinomas; (2) Metabolic disease, neurological disease, organismal injury, and abnormalities (score = 40), including genes coding for one of the indirect targets of metformin, *AMPK*, β2 non-catalytic subunit (*PRKAB2*), focal adhesion kinase (*FAK*), which is a prominent determinant in breast cancer initiation, progression, and metastasis via the maintenance of mammary cancer stem cells, beta-secretase 2 (*BACE-2*), which is a type I integral membrane glycoprotein and aspartic protease belonging to the peptidase A1 protein family, and the calpain inhibitor calpastatin (*CASP*), which plays a key, opposing role within the calpain/calpastatin system in initial tumor growth and subsequent metastastic dissemination. This gene network was identified around the amyloid precursor protein (*APP*), an androgen-induced gene associated with breast cancer cell proliferation;

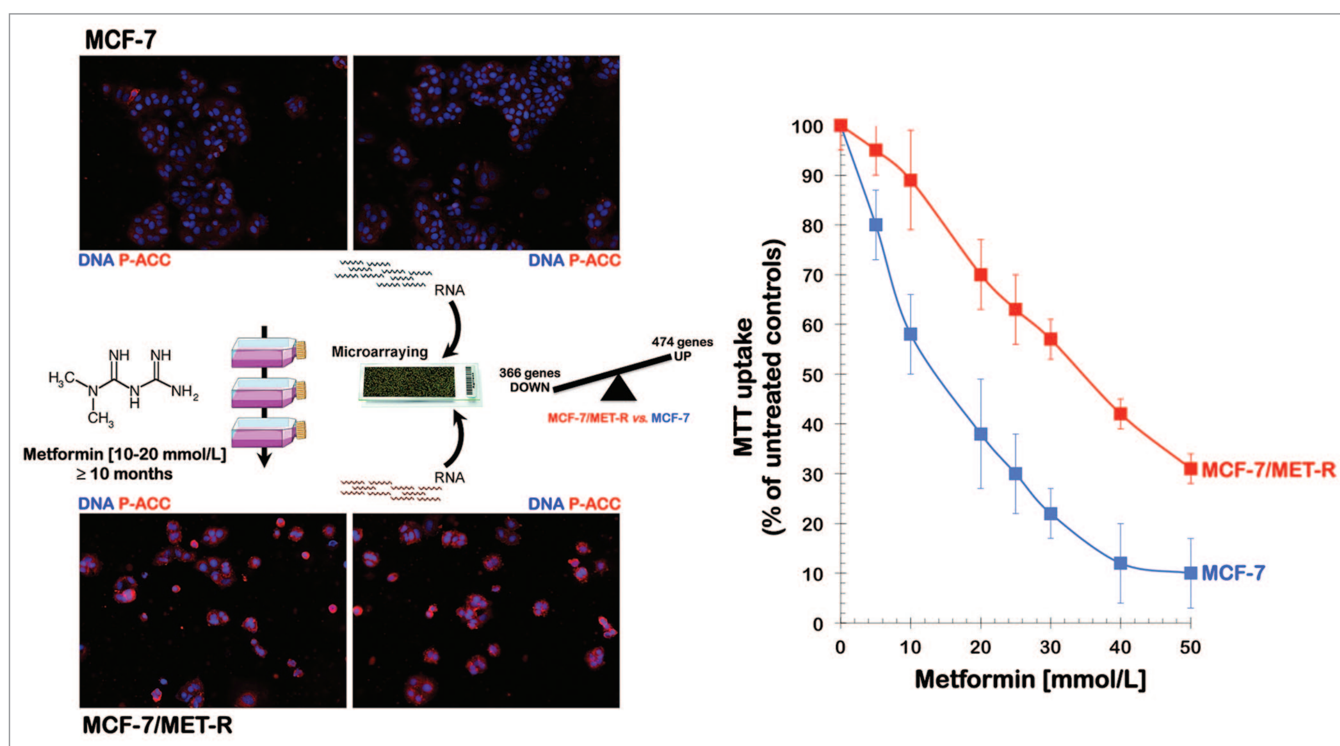

Figure 1. Discovery of a transcriptomic signature defining the acquisition of resistance to metformin. Left: A schematic depicting the experimental approach designed to establish metformin-adapted population of MCF-7 breast cancer cells. RNA was extracted from metformin-naïve MCF-7 parental cells and metformin-resistant MCF-7/MET-R cells and then hybridized to G4112F Agilent Human Whole Genome Microarrays. Gene expression was analyzed as described in the "Materials and Methods" section. For the complete gene data, see **Tables S1 and S2**. Figure shows also representative immunofluorescence images demonstrating a significant augmentation of phospho-acetyl-CoA carboxylase (P-ACC) expression, a marker of metformin-enhanced AMPK activity, as well as the reduced number and altered morphology of metformin-adapted MCF-7/MET-R cells compared with MCF-7 parental cells. Right: Figure shows dose-response MTT uptake curves confirming that MCF-7/MET-R cells exhibit increased cell viability in the presence of extremely high concentrations of metformin. Similar optical density values of MTT uptakes were obtained in untreated MCF-7 (approx. 0.8) and MCF-7/MET-R cells (approx. 0.7) after a 5-d culture period.

(3) Embryonic development, nervous system development and function, organ development (score = 37), a gene network that was identified around the *Akt* gene and included the gene coding for the transmembrane protease, serine 4 (*TMPRSS4*), which is a promoter of migration, invasion, and metastasis by facilitating EMT-like phenomena; the gene coding for the serine hydrolases monoacylglycerol lipase (*MGLL*), which is elevated in aggressive human cancer cells and plays a key role in cancer metastasis; and the genes coding for neuronal repellent Slit2 (*SLIT2*) and its transmembrane receptor *ROBO*, a key autonomous duo with oncogenic effects on tumor cells that may regulate tumorigenesis and metastasis through a mechanism related to contact inhibition. Intriguingly, this gene network included genes such as N-myc downstream regulated gene 1 (*NDRG1*), which has been shown to act as a metastatic suppressor in a number of human cancers; (4) Cellular movement, cancer, endocrine system disorders (score = 34), a gene network that was identified, at least in part, around *NUPR1*, a gene that has been found to aid the establishment of metastasis and to play a key role in the progression of several malignancies, including breast cancer, by inducing chemoresistance, protection from apoptosis, and genome instability. The other sub-network was identified around the NFkB complex and included genes such as those coding for the transmembrane mucin *MUC1*, whose overexpression is frequently associated with metastatic progression, the pro-metastatic gene *AMIGO2*, and the transcription factor *ATF3*, a molecule that functions as an integration point for cellular communication during changes in homeostasis and in the subsequent adaptation in response to those changes during breast cancer development and metastasis; (5) Cell morphology, nervous system development and function, skeletal and muscular system development and function (score = 30), a gene network that was

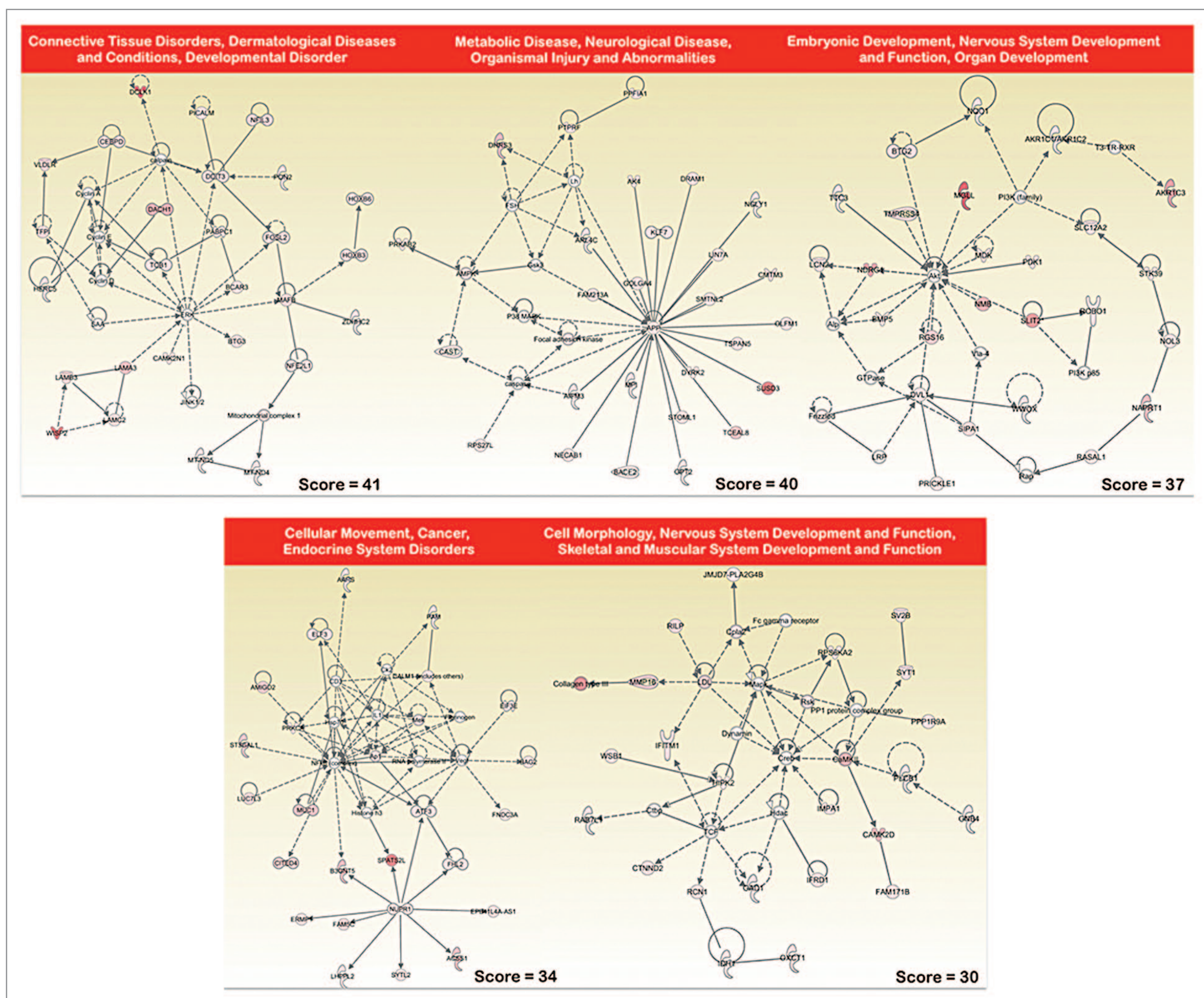

Figure 2. Network analysis of genes overexpressed in MCF-7/MET-R cells that have acquired resistance to metformin. A data set containing the differentially upregulated genes (called the focus molecules = 474) between metformin-refractory MCF-7/MET-R cells and metformin-sensitive MCF-7 parental cells was overlaid onto a global molecular network developed from information contained in the Ingenuity Pathway (IPA) Knowledge Base. Networks of these focus molecules were then algorithmically generated based on their connectivity. The figure shows upregulated networks with the 5 highest IPA scores (a composite measure that indicates the statistically significance of the interconnection between the molecules depicted in the network). The focus molecules are colored according to the gene expression (fold-change). The nodes are displayed using various shapes that represent the functional class of the gene product. Edges with dashed lines indicate indirect interactions, while continuous lines represent direct interactions.

identified around *Creb* and included genes such as those coding for the matrix metalloproteinase 16 (*MMP16*) and cytoplasmic phospholipase A2 (*Cpla2*), whose metabolites play critical roles in tumor metastasis via the promotion of angiogenesis and MMP expression. The top functions of the downregulated gene networks (**Fig. 3**) were related to: (1) Cell cycle, cellular assembly and organization, DNA replication, recombination, and repair (score = 52), a gene network that was identified around the gene coding for cyclin B1 (*CCNB1*), whose downregulation results in polyploidization during DNA damage-induced senescence, and this network included genes coding for cell cycle checkpoint proteins such as *CCNB2*, *CDC25C*, as well as spindle assembly checkpoint proteins such as *CDC20*, whose downregulation induces aberrant mitosis, the Aurora kinases *AURKA* and *AURKB*, which play important roles in chromosome alignment, segregation, and cytokinesis during mitosis, *BUB1*, whose inhibition results in genomic instability and anchorage-independent growth, and 2 out of the 3 human TACC (transforming acidic coiled-coil) genes (*TACC1*, *TACC3*) that participate in the oncogenic processes and whose downregulation alters the control of mRNA homeostasis in polarized cells; (2) Connective tissue disorders, hereditary disorder, immunological disease (score = 37), a gene network that was identified around Akt, included genes coding for semaphorins, which are a large family of secreted and membrane-bound molecules that have been found to regulate cell adhesion and cell motility, angiogenesis, immune function, and tumor progression, such as *SEMA3B*, a putative tumor suppressor gene, and *SEMA6A*, an angiogenesis inhibitor, the serine protease *HTRA1*, a tumor suppressor whose downregulation activates EMT-like phenomena and ATM DNA damage response pathways, and different types of *collagen* (IV, V, and VI); (3) Cellular assembly and organization, DNA replication, recombination, and repair, cell cycle (score = 37), a gene network that was identified around the gene coding for the anti-apoptotic protein *BRIC5* (*survivin*), the downregulation of which may allow cancer cells to exit mitosis without achieving proper chromosome alignment, leading to the formation of polyploid nuclei, and this network included genes coding for centromere and nucleosomal proteins (*CENP-M*, *CENP-A*), and members of the histone cluster 1 (*HIST1H4A*, *HIST1H2AM*); (4) Infectious disease, connective tissue disorders, developmental disorder (score = 30), a gene network that was identified around the gene

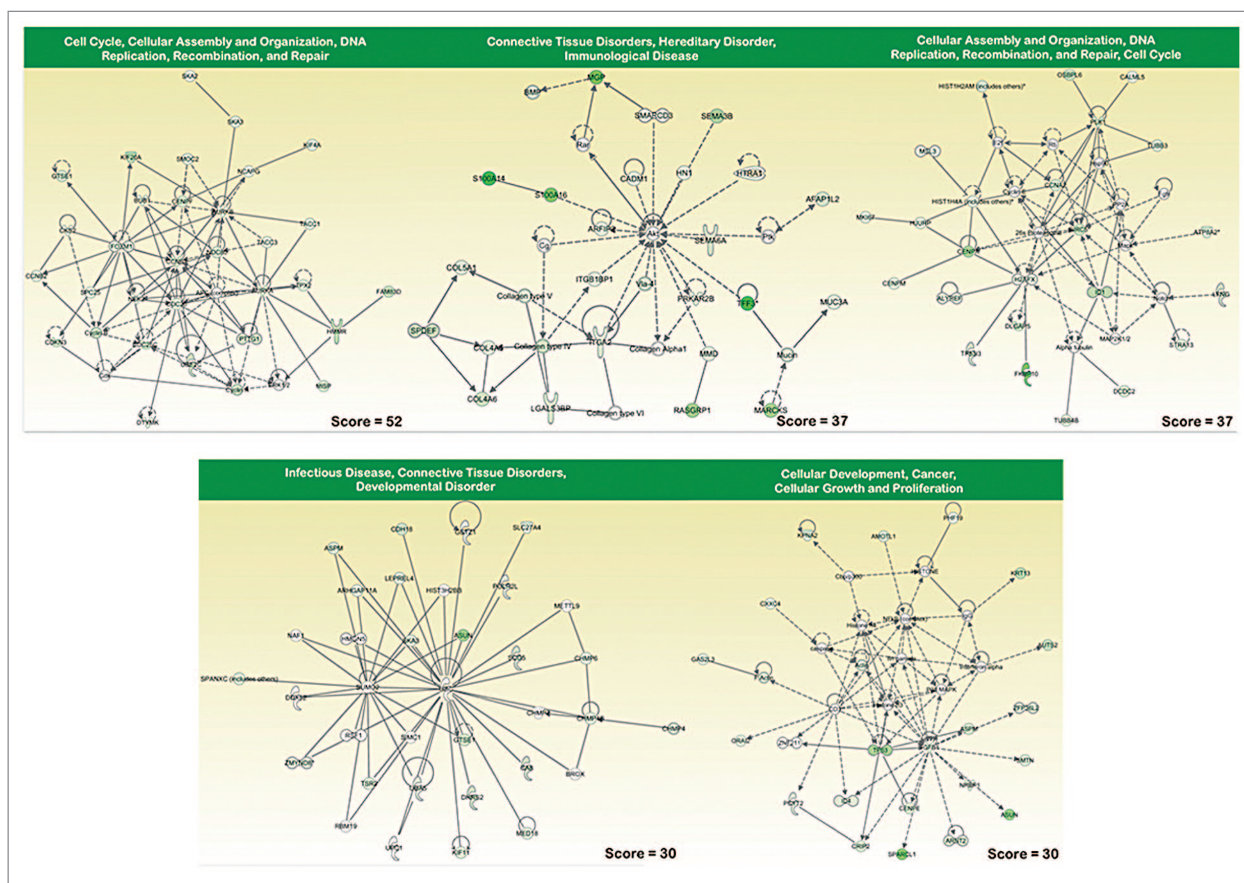

Figure 3. Network analysis of genes under-expressed in MCF-7/MET-R cells that have acquired resistance to metformin. A data set containing the differentially downregulated genes (called the focus molecules = 366) between metformin-refractory MCF-7/MET-R cells and metformin-sensitive MCF-7 parental cells was overlaid onto a global molecular network developed from information contained in the Ingenuity Pathway (IPA) Knowledge Base. Networks of these focus molecules were then algorithmically generated based on their connectivity. The figure shows downregulated networks with the 5 highest IPA scores (a composite measure that indicates the statistically significance of the interconnection between the molecules depicted in the network). The focus molecules are colored according to the gene expression (fold-change). The nodes are displayed using various shapes that represent the functional class of the gene product. Edges with dashed lines indicate indirect interactions, while continuous lines represent direct interactions.

coding for ubiquitin (UBC), and this network included several genes coding for charged multivesicular body proteins (*CHMP4*, *CHMP4B*, *CHMP6*) and the mammalian asunder gene (*ASUN*), the downregulation of which leads to nucleus–centrosome uncoupling, abnormal spindles, and multinucleation; (5) Cellular development, cancer, cellular growth, and proliferation (score = 30), a gene network that was identified around the gene coding for Histone H3 and included the gene coding for p63 (*TP63*), an "epithelial organizer" that suppresses tumorigenesis and metastasis by directly impinging on EMT, stemness, senescence, cell death, and cell cycle arrest, the Secreted protein, acidic and rich in cysteine-like 1 (*SPARCL1*), whose downregulation increases the migratory, invasive, and metastastic properties of cancer cells, and *CXXC4*, whose decreased expression promotes a malignant phenotype by activating the Wnt stemness signaling pathway.

The *ATF3* and *DDIT3* genes, 2 autophagy-related members of cell stress responses related to mTOR inhibition, and the *AMPK* gene, which codes for one of the indirect targets of metformin, were central in a merged network combining the top 5 upregulated signaling networks with the highest IPA scores in the transcriptomic signature of metformin-adapted MCF-7/MET-R cells (**Fig. 4**, top panels). The *CCNB1*, *CCNB2*, *CCNA*2, and *CDC25C* genes, all of them coding for checkpoint proteins that regulate the cell cycle, were central in a merged network combining the top 5 downregulated signaling networks with the highest IPA scores in the transcriptomic signature of metformin-adapted MCF-7/MET-R cells (**Fig. 4**, bottom panels). When the IPA software was used to determine the canonical pathway analysis enrichment categories, "chemokine signaling", "axonal guidance signaling", and "VDR/RXR activation" were the most statistically significant maps that were modulated by the upregulated genes

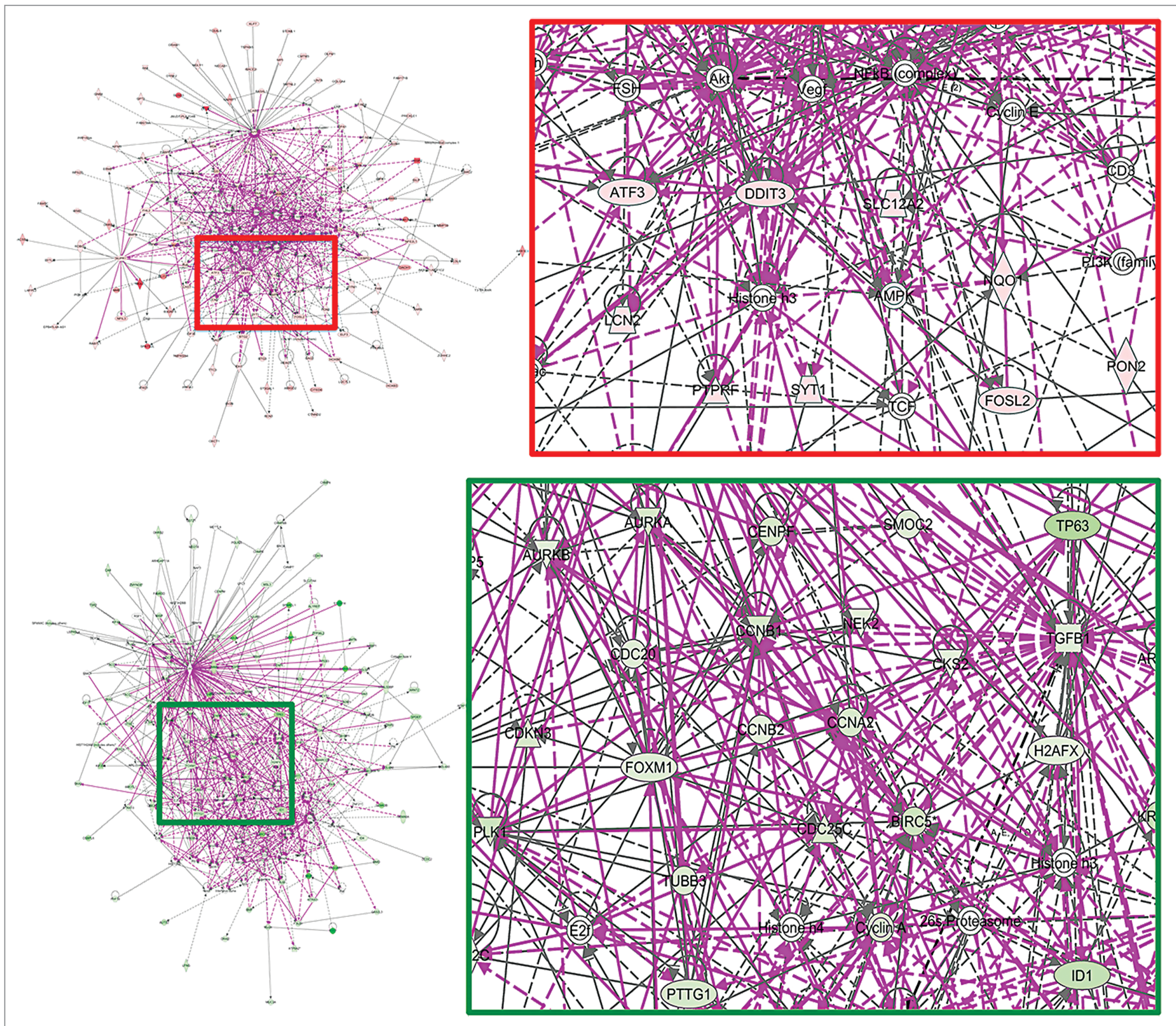

Figure 4. Merged networks combining major signaling networks associated with the transcriptomic signature of MCF-7/MET-R cells that have acquired resistance to metformin.

within the metformin-unresponsiveness transcriptomic signature (**Table 1**; **Fig. 5**, left panel). "Mitotic roles of polo-like kinases", "Axonal guidance signaling", "Cell cycle: G2/M DNA damage checkpoint regulation", and "Remodeling of epithelial adherens junctions" were the most statistically significant maps that were modulated by the downregulated genes within the metformin-unresponsiveness transcriptomic signature (**Table 1**; **Fig. 5**, right panel).

Discussion

Many genetic lesions important for cancer converge to promote proliferative metabolism in cancer cells, thus suggesting that "cancer metabolism" is a single entity that differs from "normal cell metabolism". Targeting cancer metabolism for cancer therapy has been suggested as a simpler approach than targeting the mutated gene products to eliminate all cancerous cells simultaneously. Because the extent of metabolic reprogramming that occurs in cancer cells goes far beyond glycolytic behavior (the Warburg effect) and encompasses nearly all metabolic routes, including glutaminolysis, lipogenesis, fatty acid oxidation, gluconeogenesis, and the pentose phosphate pathway, and given the extremely high metabolic flexibility of cancer cells, exclusively targeting glycolysis or specific metabolic pathways in cancer might be just as complicated as targeting somatic mutations, if not more so.[38,39]

We began to recognize that cancer cells can escape death from metabolic inhibitors by turning off the glycolytic pathway and switching to aerobic respiration and high oxidative capacity phenotype.[40] If glycolysis-addicted cancer cells can easily perform these metabolic tricks to hide among the non-proliferative oxidative phosphorylation-dependent normal cells until the treatment is over, then the possibility exists that the metabolic features of cancer cells will come back after the cessation of treatment with glycolysis inhibitors.[41] An alternative approach may involve the use of "dirty" drugs, which are able to hit several metabolic pathways simultaneously. In this regard, there is considerable excitement and an increasing number of clinical trials testing the efficacy of the anti-diabetic biguanide metformin in cancer treatment, and these trials are based on epidemiological observations linking metformin use in diabetics to reduced cancer incidence and the multi-faceted ability of metformin to redundantly reprogram energy metabolism at both the organismal and cellular levels.[10-37] Given the intrinsic metabolic flexibility of cancer cells, we recently envisioned that cancer cells could elude the metabolic stress-mediated signal transduction pathway targeted by metformin. To anticipate these obstacles, we explored the transcriptomic and signaling pathways activated upon the chronic metformin exposure of MCF-7 cells, a widely studied model for hormone-dependent human breast cancer. Our current findings establish, for the first time, that a "global" targeting of metabolic reprogramming using metformin certainly imposes great selective pressure for the emergence of resistant breast cancer cells. Intriguingly, acquired resistance to metformin in breast cancer cells appears to trigger a transcriptome reprogramming toward a degradome-related metastatic profile, as many genes encoding extracellular matrix secreted and cell membrane-associated proteases, all of which are commonly involved in cancer cell migration and invasion, were included

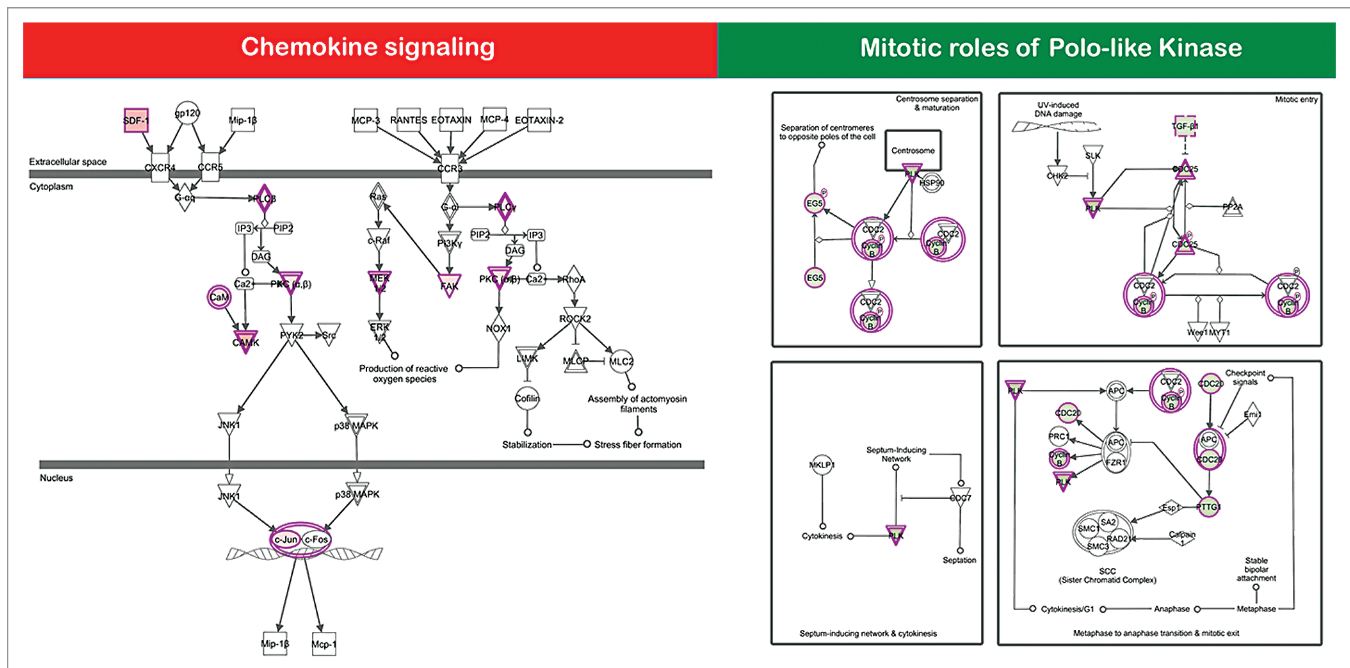

Figure 5. Canonical pathway analysis of differentially expressed genes in MCF-7/MET-R cell populations chronically adapted to grow in the presence of metformin. Using the "canonical pathways" feature of the IPA global functional analysis, we were able to identify metformin response-related pathways that were significantly impacted in metformin-refractory MCF-7/MET-R cells when compared with metformin-naïve MCF-7 parental cells. Figure shows 2 selected canonical pathways with the lowest *P* value differentially activated (left panel) or deactivated (right panel) in MCF-7 cells upon acquisition of resistance to metformin.

in the signature. These findings suggest a convergent activation of pathways underlying tumor-microenvironment interactions when the cancer cells adapt to the metabolic challenges of drugs targeting various metabolic pathways, such as the biguanide metformin.

Metformin-refractory MCF-7/MET-R cells drastically increased (30-fold upregulation vs. metformin-naïve MCF-7 cells) the expression of *KLK11* (**Table 2**), a gene encoding a cell-surface-expressed type II, trypsin-like transmembrane serine protease that was originally identified as one of the most highly upregulated genes in prostate cancer.[42-46] Kallikrein-related peptidases (KLKs) are enzymes with extracellular hydrolysis activities, such as the activation and/or degradation of their substrates, including growth factors, extracellular matrix (ECM) proteins, other cancer-associated proteases, cell membrane-bound, and adhesion proteins. These serine proteases were among the first proteolytic enzymes to be studied extensively in the "degradome", i.e., the complete set of proteases expressed at a given time within a cell, tissue, or organism.[47-51] KLK11 protein expression has been shown to be highly expressed at sites of bone metastasis and in late-stage primary tumors, suggesting a role in tumor progression. Accordingly, in vivo studies demonstrated that overexpression of *KLK11* led to tumor progression and metastasis. KLK11 expressed in ER-positive breast cancer cells, such as MCF-7, has been suggested to play a crucial role in breast cancer progression by increasing the bioavailability of insulin growth factors (IGFs) via the degradation of IGF binding protein-3 (IGFBP-3).[52]

Metformin-refractory MCF-7/MET-R cells exhibited a drastic increase (24-fold upregulation vs. metformin-naïve MCF-7 cells) in the expression of *CTSF* (**Table 2**), a gene coding for Cathepsin F, a member of the degradome cysteine proteases.[53] Although only little data are available on Cathepsin F, several human cancer cell lines have increased expression of *CTSF* compared with its normal counterpart, suggesting that this enzyme could be involved in degradative processes during tumor progression.[53-55] Cathepsins are a class of globular proteases that were initially described as intracellular peptide hydrolases, although several cathepsins also have extracellular functions. Most studies have confirmed that cathepsins are highly expressed in invasive tumors, and they mediate degradation of the ECM and collagen, increase the motility and invasion of cancer cells, mediate the dissemination of cancer cells, and induce the EMT and angiogenesis.[56-59] Cathepsins can also activate other proteases, thereby indirectly affecting invasion by participating in proteolytic cascades; moreover, cathepsins can inactivate key cell adhesion factors involved in the maintenance of the epithelial phenotype by cleaving cell surface proteins, such as E-cadherin, thus abrogating its cell–cell adhesion function and promoting tumor cell invasion.

Metformin-refractory MCF-7/MET-R cells intriguingly had increased (18-fold upregulation vs. metformin-naïve MCF-7 cells) expression of tumor necrosis factor (ligand) superfamily, member 10 (*TNFSF10*/*TRAIL*) (**Table 2**).[60-63] The protein encoded by this gene is a cytokine that belongs to the tumor necrosis factor (TNF) ligand family, which preferentially induces apoptosis in transformed and tumor cells, but does not appear to kill normal cells, even though it is expressed at a significant level in most normal tissues. However, in cells with a weak caspase-3 signaling cascade, the apoptotic effects of TNFSF10 require the caspase-8-mediated cleavage of the BH3-only BCL2/Bcl-2 family member BID to activate the intrinsic apoptosis pathway.[64] Indeed, there are mechanisms that tightly control TNFSF10-induced apoptosis, which are utilized by cancer cells to counteract the cytotoxicity of TNFSF10. In this regard, it has been reported that TNFSF10 is able to induce autophagy in certain cancer cells, protecting them by blunting the cytotoxicity of TNFSF10 and possibly contributing to TNFSF10 resistance.[65-68] The anti-apoptotic BCL2 family proteins, such as BCL2, bind beclin-1 (BECN1) to inhibit autophagy, and the dissociation of BCL2 family proteins from BECN1 promotes autophagy.[69-71] Because MCF-7 human breast carcinoma cells do not express caspase-3, earlier studies have shown that TNFSF10 induces autophagy in MCF-7 cells, and autophagy is protective against the cytotoxicity of TNFSF10 in these cells,[72] the prominent augmentation of *TNFSF10* gene expression is accompanied by a severe inhibition of *BCL2* (11-fold downregulation vs. metformin-naïve MCF-7 cells), which strongly suggests that the activation of protective autophagy plays a causative role in the acquisition of resistance to metformin. The metformin-refractory MCF-7/MET-R cells also activated (14-fold *vs*. metformin-naïve MCF-7 cells) the expression of *TNFAIP2*, which encodes tumor necrosis factor

Table 1. Top canonical pathways in the transcriptomic signature of metformin-adapted MCF-7/MET-R cells

Name	*P* value	Ratio
Chemokine signaling	5,57E-05	9/74 (0,122)
Axonal guidance signaling	3,02E-04	23/476 (0,048)
VDR/RXR activation	5,7E-04	8/87 (0,092)
CXCR4 signaling	1,66E-03	11/172 (0,064)
α-Adrenergic signaling	2,08E-03	8/106 (0,075)
Mitotic roles of polo-like kinase	1,7E-05	8/68 (0,118)
Axonal guidance signaling	3,79E-04	19/476 (0,04)
Cell Cycle: G_2/M DNA damage checkpoint regulation	1,1E-03	5/48 (0,104)
Remodeling of epithelial adherens junctions	7,28E-03	5/68 (0,074)
Role of osteoblasts, osteoclasts and chondrocytes in rheumatoid arthritis	7,68E-03	10/244 (0,041)

α (TNFα)-inducible protein 2. Similar to TNFα, which is an inflammatory cytokine that is present in the microenvironment of many tumors and is known to promote tumor progression, TNFAIP2 is a cell migration- and invasion-promoting protein, and its expression predicts shorter metastasis-free survival in cancer patients.[73]

It is worth noting that acquired resistance to metformin resulted in a drastic augmentation (12-fold upregulation in metformin-refractory MCF-7/MET-R cells vs. metformin-naïve MCF-7 cells) of *DCLK1* gene expression (**Table 2**). Doublecortin-like kinase 1 was originally described as a marker that was able to distinguish between Dclk1-positive tumor stem cells and Dclk1-negative normal stem cells in the intestine.[74] Later studies confirmed that Dclk1 regulates pluripotency and angiogenic factors via microRNA-dependent mechanisms, and its expression marks a morphologically distinct subpopulation of cells with stem cells properties in pancreatic cancer.[75,76] In this scenario, it is tempting to suggest that chronic adaptation to metformin accelerates the retrogression from a differentiated cancer cell state to a more stem-like state endowed with enhanced migratory capacities (i.e., the "migrating cancer stem cells" concept originally proposed by Thomas Brabletz).[77-80] It is also worth mentioning that the other protease gene that is notably upregulated upon acquisition of metformin resistance, in addition to *KLK11* and *CTSF*, is the gene coding for the serine hydrolase enzyme monoacylglycerol lipase (*MAGL*) (**Table 2**). MAGL is overexpressed in aggressive types of tumor cells, where it regulates a fatty acid network enriched in oncogenic signaling lipids that promote migration, invasion, survival, and in vivo tumor growth.[81-83] The overexpression of MAGL in nonaggressive cancer cells is sufficient to increase their pathogenicity by recapitulating this fatty acid network, thus revealing how cancer cells can co-opt a lipolytic enzyme to translate their lipogenic state into an array of protumorigenic signals. Indeed, MAGL's unique role of providing lipolytic sources of free fatty acids (FFAs) for the synthesis of oncogenic signaling lipids that promote cancer aggressiveness, together with the fact that MAGL blockade impairs cell migration, invasiveness, and tumorigenicity by lowering the levels of FFAs and protumorigenic signaling lipids,[84-86] strongly suggest that, in response to the expected chronic inactivation of several lipogenic enzymes and lipogenesis imposed by metformin, the metformin-refractory MCF-7/MET-R cells re-activate the very same lipogenic state that is commonly controlled by metformin's targets (AMPK, acetyl-CoA carboxylase, mTOR) via MAGL. The serine proteinase degradome gene *FREM1* (*FRAS1*-related extracellular matrix 1/signalase-like 1)[87,88] and Wnt-induced signaling protein-2 (*WISP2*/*CCN5*), a gene coding for a metalloproteinase substrate implicated in the modification of the ECM, invasion, and angiogenesis that has been linked to a variety of human cancer types and may contribute to cancer metastasis,[89,90] were also significantly upregulated in the metformin-refractory MCF-7/MET-R cells (**Table 2**).

Metformin-refractory MCF-7/MET-R cells drastically decreased (14-fold downregulation vs. metformin-naïve MCF-7 cells) the expression of *PMP22*/*GAS3* (**Table 2**), a putative tumor suppressor gene. PMP22/gas3 overexpression was originally found to induce an apoptotic-like phenotype,[91] and recent studies have revealed that the induction of *GAS3* inhibits breast cancer by inhibiting the attachment and proliferation of the tumor cells, at least in part by blocking the interaction of β1 integrin with fibronectin.[92] Indeed, the tumor-suppressive activity of *GAS3* is related to the significantly increased metastasis-free survival of breast cancer patients. Another top molecule notably decreased upon acquisition of metformin resistance was the *S100A14* (S100 calcium binding protein A14) gene (14-fold downregulation vs. metformin-naïve MCF-7 cells; **Table 2**). The levels of the protein encoded by the *S100A14* gene have been found to be lower in cancerous tissues and are associated with higher metastatic potential and advanced clinical stage, suggesting this gene has a tumor suppressor function.[93,94] The expression of the Trefoil factor 3 (*TFF3*) gene, which has been identified as a part of a gene expression signature of biologically aggressive basal-like and claudin-low breast carcinomas that are characterized by reduced expression levels or loss of epigenetic biomarker genes, such as E-cadherin and estrogen receptor, due to aberrant DNA hypermethylation,[95] was found to be notably decreased (13-fold downregulation) in the metformin-refractory MCF-7/MET-R cells compared with the metformin-naïve MCF-7 cells (**Table 2**). The MCF-7/MET-R cells notably lost (12-fold downregulation vs. metformin-naïve MCF-7 cells) the expression of the Dickkopf1 (*DKK1*) gene (**Table 2**), which encodes a secreted inhibitor of the Wnt/β-catenin pathway and may have tumor suppressor functions.[96-98] Exogenous expression of DKK1

Table 2. Top molecules in the transcriptomic signature of metformin-adapted MCF-7/MET-R cells

Molecules	Fold-change
KLK11	↑30,460
CTSF	↑24,119
TNFSF10	↑17,960
TNFAIP2	↑13,558
TMTC1	↑13,390
DCLK1	↑12,452
MGLL	↑12,360
WISP2	↑10,812
FRAS1	↑10,342
CYP1B1	↑10,268
CXorf61	↓23,062
PMP22	↓14,517
S100A14	↓14,192
TFF3	↓13,100
DKK1	↓12,359
TFF1	↓10,680
BCL2	↓10,660
CYP26B1	↓9,515
MGP	↓9,131
S100A16	↓8,254

in human malignant breast cancer cells with mesenchymal-like phenotype significantly reduces the expression of EMT-promoting factors, such as SLUG and TWIST;[99] conversely, silencing DKK1 expression in non-tumorigenic epithelial breast cells leads to increased invasive capacity and decreased E-cadherin expression.[100] Together, these findings strongly suggest that the negative effect of DKK1 on the EMT is part of the suppressive reprogramming that occurs when epithelial MCF-7 breast cancer cells adapt to the continuous presence of metformin.

Mounting evidence supports the idea that deregulated cellular metabolism is linked to drug resistance in cancer therapy.[7-19,101] Although the demonstration of resistance to oncogene-mediated targeted therapy through the adaptation of cellular metabolism suggests that the rewiring of cellular metabolism plays a fundamental, convergent role for oncogenes and signal transduction in promoting tumorigenesis, little is known about how the cancer signaling networks are remodeled and which pathways are invoked to sustain survival in the presence of drugs targeting central key signaling metabolic hubs (e.g., AMPK, mTOR) that respond to an array of signaling metabolic inputs and regulate a range of downstream effector metabolic pathways. Together, our current findings suggest, for the first time, that chronic adaptation to high doses of the AMPK agonist/mTOR inhibitor metformin appears to causally involve 2 highly intertwined molecular phenomena underlying enhanced cancer aggressiveness. On the one hand, low-proliferative MCF-7/MET-R cells appear to circumvent mitotic catastrophe-induced cell death by becoming polyploid cells and increasing genome instability; on the other hand, genomically unstable MCF-7/MET-R cells appear to simultaneously acquire a metastatic profile, as many genes encoding extracellular matrix secreted and cell membrane-associated proteases involved in cancer cell migration and invasion were included in the signature. Because adhesion-dependent loss of genomic surveillance mechanisms can significantly increase genome instability, the possibility of a reciprocal relationship exists between the activation of the cellular degradome and increased genome instability as a previously unrecognized mechanism of resistance to multi-targeted metabolic drugs, such as metformin. Indeed, it is reasonable to suggest that the unique mechanism of acquired resistance to metformin has opposing roles in growth and metastatic dissemination, while refractoriness to metformin limits breast cancer cell growth, likely due to an aberrant mitotic/cytokinetic machinery and accelerated autophagy, it notably increases the potential of metastatic dissemination by amplifying the number of pro-migratory and stemness inputs via the activation of a significant number of proteases and EMT regulators. Future studies should unambiguously elucidate whether our findings using supra-physiological concentrations of metformin mechanistically recapitulate the processes through which the induction of a migratory-stemness cellular state paradoxically occurs in a polyploid, senescent–autophagic scenario[102-105] that is triggered by the chronic metabolic stresses that commonly occur during cancer development and after treatment with cancer drugs.

Materials and Methods

Cell viability assays

The effect of metformin on cell viability was determined using a standard colorimetric 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay. For each treatment, the percent cell viability was calculated using the following equation: (OD_{570} of the treated sample/OD_{570} of the untreated sample) ×100.

Agilent gene chip analyses

Total RNA isolated from metformin-naïve MCF-7 parental cells and one pooled population of metformin-refractory MCF-7 cells (i.e., MCF-7/MET-R cells) grown in the presence of metformin was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA quantity and quality were determined using the RNA 6000 Nano Assay kit on an Agilent 2100 BioAnalyzer (Agilent Technologies) as recommended. Agilent Human Whole Genome Microarrays (G4112F) containing 45220 probes were then hybridized. Briefly, 500 ng of total RNA from each sample was amplified by Oligo-dT-T7 reverse transcription and labeled by in vitro transcription with T7 RNA polymerase in the presence of Cy5-CTP or Cy3-CTP using the Quick Amp Labeling Kit (Agilent) and purified using RNeasy columns (Qiagen). After fragmentation, 825 ng of labeled cRNA from each of the 2 samples was cohybridized in in situ hybridization buffer (Agilent) for 17 h at 65 °C and washed at room temperature (RT) for 1 min in Gene Expression Wash Buffer 1 (Agilent) and 1 min at 37 °C in Gene Expression Wash Buffer 2 (Agilent).

Statistical analysis of microarray data

The images were generated on a confocal microarray scanner (G2565BA, Agilent) at 5-μm resolution and quantified using GenePix 6.0 software (Molecular Dynamics). Spots with signal intensities that were twice that of the local background, not saturated, and not flagged by GenePix were considered reliable. Extracted intensities were background-corrected, and the log2 ratios were normalized in an intensity-dependent fashion by the global LOWESS method (intra-chip normalization). Normalized log2 ratios were scaled between arrays to allow comparisons between all data. The raw data were processed using MMARGE, a web implementation of Limma (a microarray analysis library developed within the Bioconductor Project in the R statistical environment). To identify genes that were differentially expressed, the multiclass SAM (significance analysis of microarrays) procedure was applied. Probes with Q values (FDR) below 5% and fold changes exceeding 2.0 in absolute value were initially selected as the relevant spots. The microarray probes were collapsed to genes by considering the median log2 ratio of the respective probes per gene.

Ingenuity analysis

Gene networks were constructed using Ingenuity Pathway Analysis (Ingenuity® Systems). Data sets containing identifiers of genes that were >2.0-fold up- or downregulated were uploaded into the application. These "focus genes" were overlaid onto a global molecular network developed from information

contained in the Ingenuity Pathway Knowledge Base. Networks of these "focus genes" (nodes) were algorithmically generated based on the principle that highly connected gene networks are the most biologically meaningful networks. All edges were supported by at least one reference from the literature stored in the Ingenuity Pathway Knowledge Base (the IPA interaction database is manually curated by scientists and updated quarterly). Briefly, the user-input or "focus genes" list was compared with the "global molecular network" (GMN) database, consisting of thousands of genes and interactions. The focus genes were sorted based on highest to lowest connectivity within the GMN, and networks of approximately 35 genes were grown starting with the most connected focus gene. IPA assigns a P value for a network of size n and an input focus gene list of size f by calculating the probability of finding f or more focus genes in a randomly selected set of n genes from the GMN. The intensity of the node color indicated the degree of expression (green scale for downregulated nodes; red scale for upregulated nodes). The nodes were displayed using various shapes, each of which represents a functional class of the gene products. The score indicated the likelihood of the genes in a network being found together due to random chance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/27982

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