Metformin inhibits the inflammatory response associated with cellular transformation and cancer stem cell growth

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Metformin, the first-line drug for treating diabetes, inhibits cellular transformation and selectively kills cancer stem cells in breast cancer cell lines. In a Src-inducible model of cellular transformation, metformin inhibits the earliest known step in the process, activation of the inflammatory transcription factor NF-κB. Metformin strongly delays cellular transformation in a manner similar to that occurring upon a weaker inflammatory stimulus. Conversely, inhibition of transformation does not occur if metformin is added after the initial inflammatory stimulus. The antitransformation effect of metformin can be bypassed by overexpression of Lin28B or IL1β, downstream targets of NF-κB. Metformin preferentially inhibits nuclear translocation of NF-κB and phosphorylation of STAT3 in cancer stem cells compared with non-stem cancer cells in the same population. The ability of metformin to block tumor growth and prolong remission in xenografts in combination with doxorubicin is associated with decreased function of the inflammatory feedback loop. Lastly, metformin-based combinatorial therapy is effective in xenografts involving inflammatory prostate and melanoma cell lines, whereas it is ineffective in noninflammatory cell lines from these lineages. Taken together, our observations suggest that metformin inhibits a signal transduction pathway that results in an inflammatory response. As metformin alters energy metabolism in diabetics, we speculate that metformin may block a metabolic stress response that stimulates the inflammatory pathway associated with a wide variety of cancers.

cancer treatment | inflammation | cancer prevention

Epidemiological studies show that diabetes is correlated with increased cancer risk (1, 2) and that diabetics treated with metformin have reduced risk of developing various types of cancer (3, 4). In addition, mRNA profiling of two isogenic models of cellular transformation defined a transcriptional signature that links multiple types of cancer with diabetes and other metabolic diseases (5). Metformin is used extensively to treat individuals with type 2 diabetes, obesity, and polycystic ovarian syndrome. Metformin inhibits growth of breast cancer cell lines (6–8), blocks transformation in an inducible model system (5, 9), and selectively inhibits the growth of cancer stem cells (CSCs) in genetically distinct breast cancer cells lines (9).

In mouse xenografts involving a human breast cancer cell line, the combination of metformin and doxorubicin increases the rate of tumor regression compared with treatment with doxorubicin alone, and this combinatorial therapy prevents relapse for at least several months (9). Metformin works in combination with other standard chemotherapeutic drugs (paclitaxel and cisplatin), and it has comparable effects on tumor regression and preventing relapse when metformin combined with a fourfold reduced dose of doxorubicin that is not effective as a monotherapy (10). Lastly, the combination of metformin and doxorubicin prevents relapse in xenografts generated with prostate and lung cancer cell lines (10).

The mechanism of metformin action has been studied primarily in the context of diabetes, and it is poorly understood. Metformin inhibits glucose production in the liver, and it activates AMP kinase (AMPK), which plays a key role in insulin signaling and energy sensing (11). AMPK activation requires LKB1, a protein kinase that is a tumor suppressor associated with Peutz–Jeghers syndrome (12, 13). However, the AMPK/LBK1 pathway is not required for the metabolic effects of metformin on glucose uptake (14), indicating that AMPK is merely an associated characteristic of metformin treatment and reduced glucose uptake. In this regard, metformin inhibits mTORC1 in the absence of AMPK and TSC2 (15, 16). Metformin also regulates the expression of certain miRNAs and CSC-specific genes (17), but it is unclear which if any of these are direct targets. There is some evidence that metformin might directly affect complex 1 of the mitochondrial respiratory chain (18, 19) and that this inhibition might be responsible for the increased AMPK activity. However, metformin inhibition of complex 1 function in isolated mitochondria requires very high metformin concentrations (18). In the context of atherosclerosis, metformin inhibits NF-κB activation and decreases C-reactive protein levels (20), and it inhibits the inflammatory response via a pathway involving AMPK and the tumor suppressor PTEN (21). Not much is known about how metformin mediates any of its anticancer effects.

In previous work, we described an inducible model of breast cell transformation (5) in which a transient inflammatory signal initiates an epigenetic switch from nontransformed to cancer cells (22). This epigenetic switch is mediated by an inflammatory positive feedback loop involving the NF-κB and STAT3 transcription factors, Lin28B, IL6, microRNAs (Let-7, miR-181b, miR-21), and tumor suppressor genes (PTEN and CYLD) (22, 23). The transformed cells contain a minority population of CSCs that have an enhanced inflammatory loop that results in overproduction of IL6 (22, 24). The CSCs and non-stem cancer cells (NSCCs) within the transformed population are in a dynamic equilibrium that involves IL6 secretion (25) and a transcriptional regulatory circuit that acts as a bistable switch and involves the miR-200 family, several other miRNAs, the Zeb repressors, the Klf4 activator, and polycomb complexes (26).

Here, we use this inducible model of cellular transformation to investigate the mechanism of metformin in a cancer context. Taken together, our observations suggest that metformin inhibits the inflammatory pathway necessary for transformation and CSC formation. To link our results with previous work on metformin in the diabetic context, we speculate that metformin may block a metabolic stress response that stimulates the inflammatory pathway associated with a wide variety of cancers.

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Results

Metformin Blocks Src-Induced Cellular Transformation at an Early Stage. In our inducible model of cellular transformation, treatment of nontransformed mammary epithelial cells (MCF-10A) containing ER-Src with tamoxifen rapidly induces Src, and morphological transformation is observed within 24–36 h (9, 22). Transcriptional profiling reveals a rapid inflammatory response, with activation of NF-κB being observed within 15 min. A few hours thereafter, Let-7 miRNA levels start decreasing, resulting in a 10–20-fold decrease after 24–36 h. In this model, a low dose of metformin (0.1 or 0.3 mM) that does not affect the growth of nontransformed cells blocks morphological transformation, and it also inhibits invasive growth in wound-healing assays, focus formation, and formation of colonies in soft agar (5, 9).

We first analyzed genome-wide transcriptional profiles in cells treated for 24 h with metformin, tamoxifen, or both. The transcriptional profile of cells treated with both tamoxifen and metformin is similar to that of untreated cells, suggesting that metformin reverses most (and perhaps all) of the gene expression changes mediated by Src induction. More directed analyses of inflammatory genes at the 4 h time point indicates that metformin inhibits the Src-inducible expression (Fig. 1*A*). Similarly, metformin reverses the induction of Lin28B (Fig. 1*B*) and the down-regulation of most (but not all) Let-7 miRNA family members (Fig. 1*C*), the production of inflammatory molecules (IL-1α, IL-1β, IL-6, and VEGF; Fig. 1*D*), and the level of phosphorylated STAT3 (Fig. S1*A*), the active form of this transcription factor. Lastly, metformin inhibits NF-κB activity, phosphorylation of IkB-Ser32, and expression of an NF-κB reporter construct (Fig. S1 *B–D*). Taken together, these results suggest that metformin blocks transformation by inhibiting NF-κB activation, the earliest known step in the process.

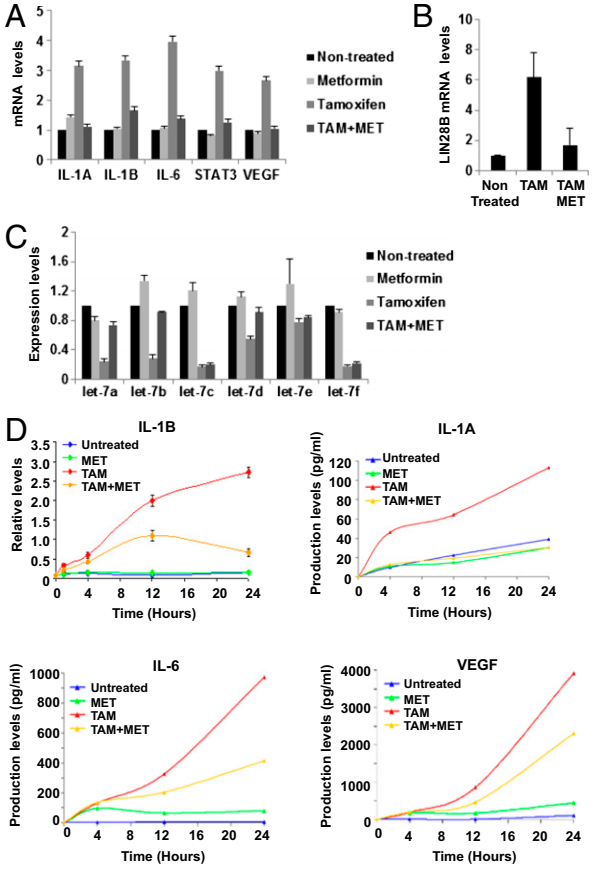

Fig. 1. Metformin reverses the inflammatory response during inhibition of cellular transformation. (*A*) mRNA levels of inflammatory genes, (*B*) Lin28B, and (*C*) let-7 family members in tamoxifen- and/or metformin-treated ER-Src cells assessed by real-time PCR. (*D*) IL1β, IL1α, IL6, and VEGF levels in tamoxifen and/or metformin treated cells assayed by ELISA.

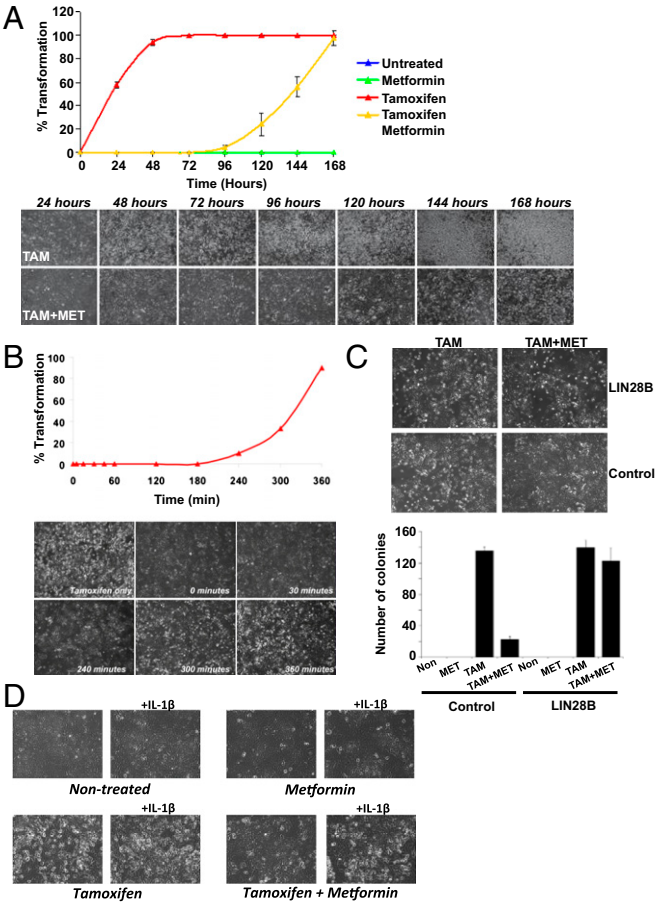

Fig. 2. Early effects of metformin to suppress cellular transformation. (*A*) Percentage and morphology of transformed ER-Src cells after treatment with tamoxifen and/or metformin. (*B*) Percentage and morphology of ER-Src cells treated with metformin at different times after tamoxifen induction. (*C*) Morphology and number of colonies of ER-Src cells treated with tamoxifen and tamoxifen and metformin in the presence or absence of a Lin28B expression vector. (*D*) Morphology of tamoxifen- and/or metformin-treated ER-Src cells treated with IL-1β.

Metformin Delays, but Does Not Completely Block, the Transition Between the Nontransformed and Transformed States. In our inducible model of cellular transformation, the duration of tamoxifen treatment is inversely related to the time needed to achieve the transformed state (22). For example, cells can be stably transformed after only 5 min of tamoxifen treatment, but the process takes 72 h as opposed to the usual 24 h. As our previous observation that metformin blocks transformation was based on assays lasting 24–36 h, we examined the effect of metformin on a longer time frame. Based on a morphological assay, cells do not appear to be transformed after treatment with tamoxifen and metformin for 4 d (Fig. 2*A*). However, morphological changes are observed after 5 d, and virtually all cells appear transformed after 7 d. Thus, metformin does not completely block cellular transformation, but rather severely delays the process. In this sense, the effect of metformin is similar to that of reducing the inducing

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signal, suggesting that metformin blocks a Src-dependent pathway that initiates the transformation process.

Metformin Does Not Inhibit Transformation When Added After the Initial Inflammatory Stimulus. The ability of metformin to inhibit virtually the entire transcriptional response and to behave in a manner similar to reducing the inducing signal suggests that metformin blocks cellular transformation at an early stage when the initial inflammatory response is triggered. To provide additional evidence for this suggestion, we monitored transformation after adding metformin at various times after the process was induced with tamoxifen (Fig. 2*B*). Metformin appears to completely block transformation (assayed at 36 h) even when added 3 h after tamoxifen addition, whereas it has little effect when added 6 h after induction (partial inhibition is observed when added 4 or 5 h after induction). Thus, metformin inhibits transformation only when added at the early stage of the process.

Expression of Lin28 or IL-1β Bypasses the Metformin Block of Transformation. As metformin appears to block the transformation process at an early stage, we examined whether expression of components induced upon tamoxifen addition could bypass the effect of metformin on transformation. We tested Lin28 and IL-1β, both of which are rapidly induced and important for transformation. Overexpression of Lin28 via an expression plasmid (Fig. 2*C*) or addition of IL1β (Fig. 2*D*) strongly inhibits the effect of metformin on transformation. These observations provide additional evidence that metformin blocks transformation at an early stage.

Metformin Selectively Inhibits the Inflammatory Signature in CSCs. In our inducible model, ~10% of the transformed cells behave as "CSCs" as defined by expression of cell surface markers ($CD44^{high}/CD24^{low}$), ability to form mammospheres, high tumor formation in xenografts, and resistance to standard chemotherapeutic drugs (22, 25). Despite their name, these CSCs do not represent a stable epigenetic state, but rather are in dynamic equilibrium with the majority population of NSCCs via secretion of IL6 and perhaps other molecules (25). Among other differences, CSCs have an enhanced inflammatory regulatory circuit (i.e., higher levels of NF-κB activation, higher levels of IL6 and other inflammatory molecules, and lower Let-7) compared with NSCCs (22).

Metformin selectively kills CSCs and, as such, acts together with chemotherapy to inhibit tumor growth and prolong remission in mouse xenografts (9, 10). Given that CSCs have an enhanced inflammatory regulatory circuit (i.e., higher levels of NF-κB activation, higher levels of IL6 and other inflammatory molecules, and lower Let-7) compared with NSCCs (22), we examined the effect of metformin on various aspects of the inflammatory signature in CSCs and NSCCs (Fig. 3). In CSCs, metformin significantly inhibits expression of a variety of inflammatory genes (Fig. 3*A*), Lin28B gene expression (Fig. 3*B*), and VEGF protein expression (Fig. 3*C*). In all cases, metformin treatment results in levels of expression that are comparable to those seen in the corresponding NSCCs.

Metformin Selectively Inhibits NF-κB Nuclear Localization and STAT3 Activity in CSCs. We also examined the effect of metformin on NF-κB and STAT3 activity in CSCs and NSCCs (Fig. 3 *D* and *E*). In CSCs, metformin reduces NF-κB levels in the nucleus and

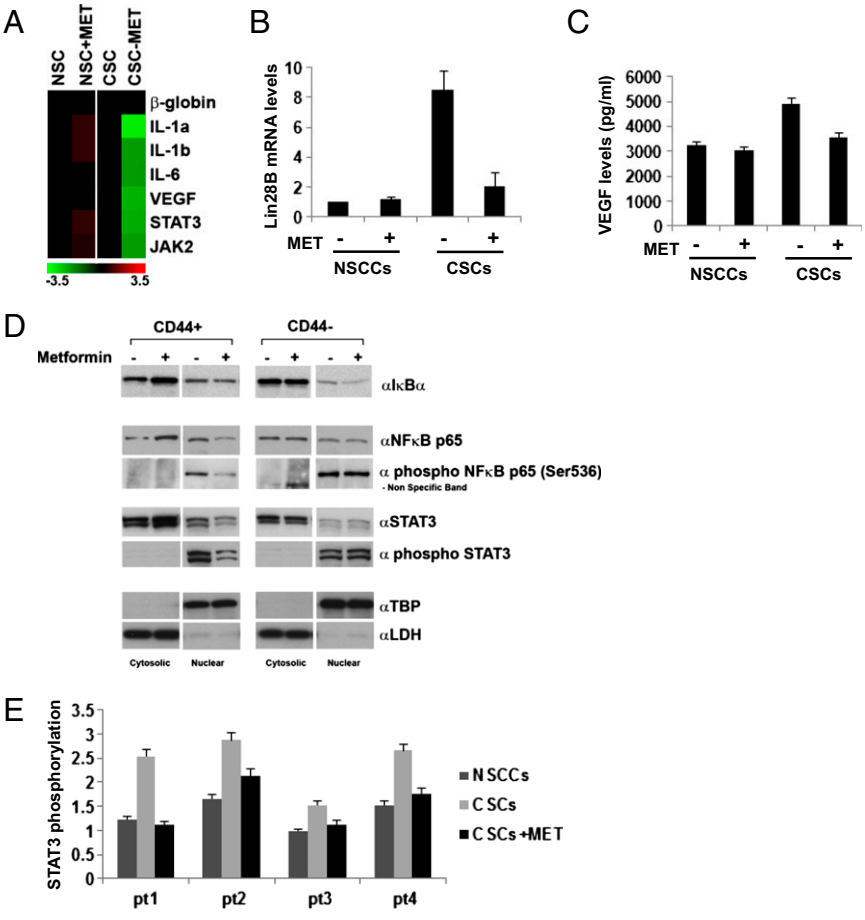

Fig. 3. Metformin inhibits the inflammatory response in CSCs. (*A*) Heat map representation of mRNA levels of the indicated inflammatory genes. (*B*) Lin28B mRNA levels. (*C*) VEGF levels in NSCCs or CSCs derived from ER-Src transformed cells treated with metformin. (*D*) Western blot analyses of the indicated proteins in cytosolic and nuclear fractions of $CD44^{+}$ and $CD44^{-}$ ER-Src cells treated with metformin. TBP and LDH protein levels were used as loading controls. (*E*) STAT3 phosphorylation levels in NSCCs and CSCs derived from four human breast cancer tissues (pt 1–4).

increases NF-κB levels and phosphorylation of IκB in the cytoplasm, whereas metformin has no such effects in NSCCs. These observations indicate that metformin selectively affects nuclear localization, and hence transcriptional activity, via IκB phosphorylation in CSCs. Similarly, metformin selectively inhibits STAT3 phosphorylation in CSCs. The inhibitory effects on STAT3 are also observed in CSCs derived from human breast tumors (Fig. 3*E*).

Combinatorial Effects of Metformin, in Combination with Doxorubicin, in Mouse Xenografts Are Associated with Inhibition of the Inflammatory Feedback Loop. In previous work, we showed that the combination of metformin with doxorubicin or other standard chemotherapeutic agents accelerates tumor regression and prolongs remission in mouse xenografts (9, 10). In addition, metformin decreases the dose of chemotherapy necessary for these anticancer effects, and it is effective in combination with concentrations of doxorubicin that are marginally effective as a monotherapy (10). All of these metformin-dependent effects in mouse xenografts are associated with selective killing of CSCs in the tumors.

We therefore analyzed components of the inflammatory feedback loop necessary for the epigenetic switch involved in cellular transformation (22) in the context of metformin-based combinatorial therapy in mouse xenografts (Fig. 4). In combination with normal (4 mg/kg) or reduced (1 mg/kg) doxorubicin, metformin causes decreased expression of Lin28B and IL6 as well as increased expression of Let-7 (Fig. 4*C*). Mice treated with doxorubicin alone have levels of these components that are comparable to those of untreated mice. In addition, AKT phosphorylation is significantly reduced in xenografts treated with metformin-based combinatorial therapy compared with doxorubicin alone (Fig. 4*D*). Thus, metformin inhibits the inflammatory feedback loop in mouse xenografts, and this inhibition is associated with CSC loss and prolonged remissions.

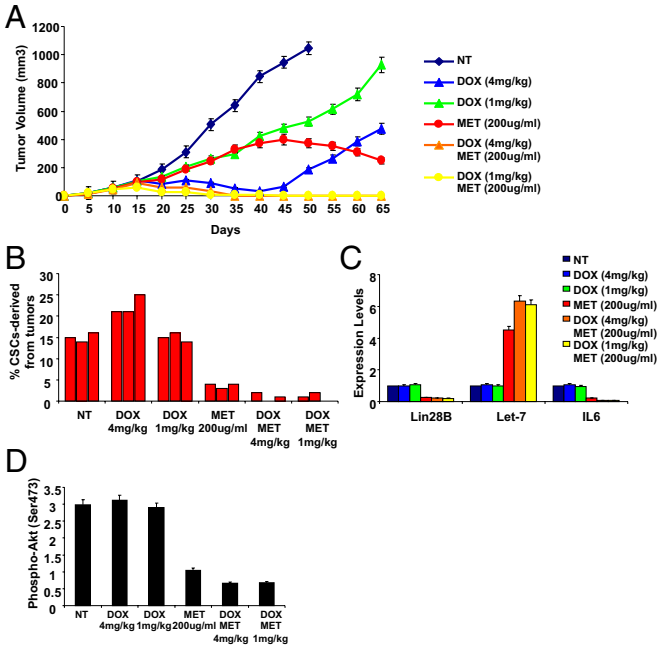

Fig. 4. Metformin prolongs tumor relapse in xenografts through suppression of the epigenetic switch. (*A*) Tumor volume (mm^3) of ER-Src transformed cells treated with doxorubicin (DOX), metformin (MET), and their combination. (*B*) Percentage of CSCs in xenograft tumors described above at day 25. (*C*) RNA levels of the indicated genes. (*D*) AKT phosphorylation levels (Ser473) in CSCs derived from xenograft tumors at day 25.

Metformin-Based Combinatorial Therapy Is More Effective in Xenografts Involving Highly Inflammatory Cell Lines. The above results linking the effects of metformin to inhibition of the inflammatory pathway suggest the metformin-based combinatorial therapy might be more effective in xenografts involving cell lines with a strong inflammatory signature. We tested this possibility by examining tumor growth in xenografts involving inflammatory and noninflammatory prostate, melanoma, and liver cancer cell lines. As shown in Fig. 5, tumors generated by inflammatory cell lines are essentially eliminated by the combination of metformin and doxorubicin, whereas tumors generated by their noninflammatory counterparts were largely unaffected.

Discussion

Evidence That Metformin Inhibits the Inflammatory Pathway. Numerous studies on metformin's mode of action have been performed in the context of diabetes given the widespread and long-standing use of metformin to treat this disease. Metformin treatment results in many biochemical changes (e.g., up-regulation of AMPK), but it has been unclear which if any of these effects are direct. The best evidence for a direct metformin target is complex 1 of the mitochondrial respiratory chain, because effects can be seen in isolated mitochondria (18, 27), but even this must be considered a preliminary indication rather than a convincing demonstration. The relevance of these diabetes-based studies of metformin action to its role as an anticancer drug is unclear.

In an inducible model of cellular transformation and CSC formation, metformin severely inhibits the epigenetic switch between nontransformed and transformed cells, and it selectively inhibits the growth of CSCs as opposed to NSCCs obtained from the same population (5, 9). Here, we provide multiple lines of evidence suggesting that these apparently diverse effects can be explained by metformin inhibiting the inflammatory pathway. First, transcriptional profiling indicates that metformin essentially blocks all Src-dependent effects on gene expression, and the inflammatory response is the earliest observable step in the transformation process initiated by Src induction (5, 22). Second, metformin severely delays but does not completely block transformation, and the time needed for transformation is inversely correlated with the duration of the tamoxifen induction step, and hence the strength of the inflammatory stimulus (22). Third, metformin blocks transformation much more effectively when added at or near the time of the initial inflammatory stimulus than at later times, indicating a primary inhibitory effect early in the transformation process. Fourth, metformin preferentially inhibits NF-κB translocation via IkB phosphorylation in CSCs versus NSCCs, and the inflammatory positive feedback loop necessary for transformation is more pronounced in CSCs than in NSCCs (22). Fifth, the inflammatory feedback loop in CSCs isolated from mouse xenografts treated with a combination of metformin and doxorubicin is strongly inhibited compared with CSCs isolated from xenografts treated with either drug alone. Sixth, although limited to only two examples, inflammatory prostate and melanoma cancer cell lines are more susceptible to metformin-based combinatorial therapy than noninflammatory cancer cell lines from the same developmental lineage. Thus, our results provide strong evidence that the anticancer effects of metformin involve inhibition of the inflammatory pathway, although they do not identify the direct molecular target in this process.

Mechanistic Implications. Metformin severely inhibits the Src-mediated induction of the inflammatory pathway, as defined by activation of NF-κB via phosphorylation of IκB. As such, metformin presumably inhibits a step(s) in the signal transduction pathway between Src and phosphorylation of IκB. The details of this pathway are poorly understood, and there are numerous environmental signals, genetic events, and molecular pathways

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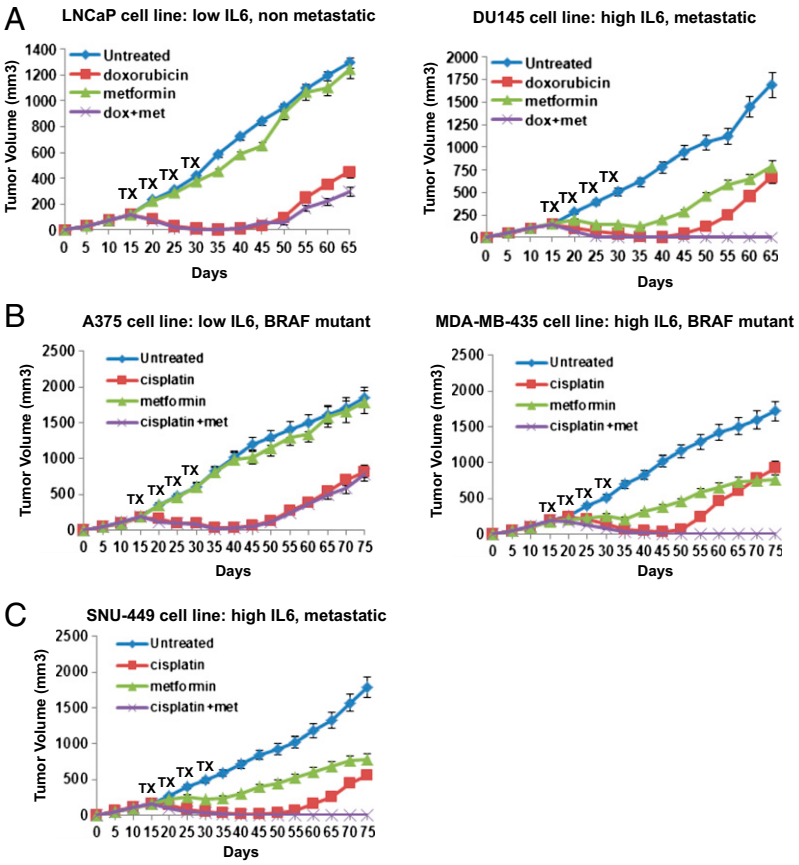

Fig. 5. Metformin effects as a monotherapy or in combination with chemotherapy to suppress the growth of inflammatory-related xenograft tumors. Tumor volume (mm^3) of xenografts with the indicated (*A*) prostate, (*B*) melanoma, and (*C*) liver cell lines treated with metformin and/or chemotherapy.

that lead to IκB phosphorylation and the resulting activation of NF-κB. In this sense, NF-κB is analogous to the yeast Msn2 and Msn4 transcriptional activator proteins that are activated by numerous stress conditions via multiple signal transduction pathways coalescing on protein kinase A (28, 29).

The epidemiological and molecular links between diabetes and cancer make it tempting to link the anticancer effects of metformin with its antidiabetic effects. In this regard, inflammation plays a key role in both cancer and diabetes, and altered glucose and energy metabolism (i.e., the Warburg effect) is a major feature of cancer cells (30–32). In addition, lipid metabolism is altered both in diabetes and cancer, and it is important for cellular transformation in the model system used in this study (5). We therefore suggest that metformin targets a component(s) of a signal transduction pathway that is triggered by metabolic stress and is ultimately transmitted to NF-κB and a subsequent inflammatory response. By blocking this component(s), metformin inhibits inflammation and aspects of glucose and energy metabolism, and it is possible that additional links between inflammation and metabolism may reinforce the effects of metformin. In this view, Src is either a component of the metabolic-stress pathway, or oncogenic v-Src ectopically stimulates some component(s) in the pathway.

Clinical Implications. There is considerable excitement and an increasing number of clinical trials testing the efficacy of metformin in cancer treatment, which are based on epidemiological observations linking metformin use in diabetics to reduced cancer incidence (3, 4), and the ability of metformin to selectively inhibit CSC growth and act together with standard chemotherapy to prolong remission in mouse xenografts (9, 10). The observation that metformin inhibits the inflammatory pathway prompts the issue of whether this drug would affect the immune system. In this regard, inflammatory-based cancer treatments such as antibodies against IL6 have proven problematic in balancing anticancer effects with immune deficiency.

Metformin has been used to treat hundreds of millions of diabetics, and it does not appear to have any effect on the classic inflammatory response that underlies a functional immune system. We suggest that the metabolic stress-mediated signal transduction pathway targeted by metformin differs from the signal transduction pathway in B and T cells that mediate the immune response. In other words, metformin does not unilaterally inhibit NF-κB function, but rather only does so by blocking a specific signal transduction pathway linked to glucose and anabolic metabolism that ultimately activates NF-κB. In this regard, metformin improves CD8 T-cell memory by modulating fatty acid metabolism (33). We suspect that this glucose- and metabolism-mediated pathway operates in many different cell types, and hence might explain why metformin reduces incidence of different human cancers and why the combination of metformin and chemotherapy is effective on many cell types in the xenograft context. While this pathway is hypothetical and has not been described in molecular terms, our results suggest that components in this pathway might be potential targets for cancer therapy.

Materials and Methods

Cell Culture. MCF10A-ER-Src cells were cultured as described previously (22) and induced to transform with 1 μM 4OH-tamoxifen (H7904, Sigma) in ethanol. Transformation occurred 36 h posttamoxifen treatment.

Chemicals. Metformin (Sigma) was dissolved in water and typically added to 0.1 mmol/L. Doxorubicin (D1515, Sigma) was dissolved in DMSO, and cisplatin (P4394, Sigma) was dissolved in water.

RNA Analysis. mRNA expression analysis for interleukins, Lin28B, and let-7 family members has been performed by real-time PCR using primers described previously (22).

Western Blot Analysis. The protein levels of IκBα (4812, Cell Signaling Inc.), NF-κB, phosphorylated NF-κB (3033, Cell Signaling Inc.), STAT3 (9139, Cell Signaling Inc.), and phosphorylated STAT3-Tyr705 (9183, Cell Signaling Inc.) were analyzed by Western blotting using cytosolic and nuclear lysates purified from $CD44^+$ ER-Src transformed cells treated with metformin. The $CD44^+$ fraction of ER-Src transformed cells was purified by magnetic sorting (PLS4948, R&D Systems).

ELISA Assay. The levels of IL1β, IL1α, IL6, and VEGF were analyzed by ELISAs as described previously (22).

Colony Formation Assay. Triplicate samples of 5×10^4 cells were treated with metformin and/or tamoxifen in the presence of the Lin28 expression vector and were mixed 4:1 (vol/vol) with 2% agarose in MCF-10A growth medium for a final concentration of 0.4% agarose. The cell mixture was plated on top of a solidified layer of 0.5% agarose in growth medium. Cells were fed every 6–7 d with growth medium containing 0.4% agarose. The number of colonies was counted after 15 d. The experiment was repeated thrice, and the statistical significance was calculated using Student *t* test.

Xenograft Experiments. MCF10A-ER-Src cells (5×10^6) were injected into the right flank of 18 female nu/nu mice (Charles River Laboratories), all of which developed tumors in 10 d with a size of ~100 mm^3. The mice were randomly distributed into six groups (three mice/group) that were untreated or treated by intratumoral injections every 5 d (four cycles) with 1 mg/kg or 4 mg/kg doxorubicin, 200 μg/mL metformin (diluted in the drinking water), or the combination. In another experiment, LNCaP and DU145 prostate cancer cells (5×10^6) were injected into the right flank of 12 female nu/nu mice, all of which developed tumors in 10 d with a size of ~75 mm^3. The mice were randomly distributed into four groups that were untreated or treated by intratumoral injections every 5 d (four cycles) with 4 mg/kg doxorubicin and/or 200 μg/mL metformin. In another experiment, A375 and MDA-MB-435 melanoma cells (7×10^6) were injected into the right flank of 12 female nu/nu mice, all of which developed tumors in 10 d with a size of ~50 mm^3. The mice were randomly distributed into four groups that were untreated or treated by intratumoral injections every 5 d (four cycles) with 10 mg/kg cisplatin and/or 200 μg/mL metformin. Finally, SNU-449 liver cancer cells (10^7) were injected into the right flank of 12 female nu/nu mice, all of which developed tumors in 10 d with a size of ~50 mm^3. The mice were randomly distributed into four groups that were untreated or treated by intratumoral injections every 5 d (four cycles) with 10 mg/kg cisplatin and/or 200 μg/mL metformin. Tumor volume (mean ± SD) was measured at various times after the initial injection.

Human Breast Tissues. STAT3 phosphorylation (Tyr705) levels were assessed by ELISA (4607, R&D Systems Inc.) in CSCs purified from four human breast tissues as described previously (23, 34). Briefly, the tissues were maintained in 10% (vol/vol) DMSO, and the CSC purification process was initiated by thawing the cryovials rapidly in a water bath at 37 °C for 50–60 s. Next, the tissues were rehydrated in a two-step process. Initially, the tissues were placed in 90% knockout serum replacement (10828010, Gibco Inc.) and 10% DMSO followed by placing the tissues in knockout DMEM (10829-018, Invitrogen) plus 10% knockout serum replacement without DMSO. The enzymatic digestion of the tissues was followed by magnetic sorting as described above.

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