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Diabetes-associated breast cancer is molecularly distinct and shows a DNA damage repair deficiency

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Running title: Diabetes and breast cancer

Key words: Breast cancer, diabetes, metabolome, transcriptome, metastasis, DNA repair, mutational signature, health disparity

Abbreviations: 1,5-AG, 1,5-anhydroglucitol; CML, N-(1-carboxymethyl)-L-lysine; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; FDR, false discovery rate; GSEA, gene set enrichment analysis; PCA, principal component analysis; ROS, reactive oxygen species.

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ABSTRACT (152 words)
Diabetes commonly affects cancer patients. We investigated the influence of diabetes on breast cancer biology using a three-pronged approach that included analysis of orthotopic human tumor xenografts, patient tumors, and breast cancer cells exposed to diabetes/hyperglycemia-like conditions. We aimed to identify shared phenotypes and molecular signatures by investigating the metabolome, transcriptome, and tumor mutational burden. Diabetes and hyperglycemia did not enhance cell proliferation but induced mesenchymal and stem cell-like phenotypes linked to increased mobility and odds of metastasis. They also promoted oxyradical formation and both a transcriptome and mutational signatures of DNA repair deficiency. Moreover, food- and microbiome-derived metabolites tended to accumulate in breast tumors in the presence of diabetes, potentially affecting tumor biology. Breast cancer cells cultured under hyperglycemia-like conditions acquired increased DNA damage and sensitivity to DNA repair inhibitors. Based on these observations, we conclude that diabetes-associated breast tumors may show an increased drug response to DNA damage repair inhibitors.

SUMMARY (25 word limit)
Diabetes-associated breast cancer is molecularly distinct and may show an increased sensitivity to DNA damage response inhibitors that are cancer therapeutics.
INTRODUCTION

Comorbidities like diabetes adversely affect cancer patients with an increasing frequency (1, 2). They negatively and disproportionately impact underserved populations and may alter tumor biology and metastasis and the choice of treatment (3). Diabetes in breast cancer patients is linked to an increased mortality (4, 5). In African American women, diabetes associated with decreased breast cancer survival in patients independent of the tumor estrogen receptor (ER) status (6).

Diabetes is thought to promote cancer development and progression through hyperglycemia, altered insulin signaling, and excessive inflammation (7, 8). Metabolic health, rather than obesity, might be relevant for breast cancer risk stratification (9). Although studies that investigated the diabetes-induced tumor biology in breast cancer patients remain sparse (10), multiple investigations have described the impact of hyperglycemia and diabetes in mouse models of breast cancer (10-14). In the 4T1 mouse model of breast cancer metastasis, hyperglycemia impaired tumor vascularization but enhanced metastatic seeding due to impaired secretion of granulocyte colony-stimulating factor and impaired neutrophil mobilization at the metastatic site (12). Other observations show that diabetes and hyperglycemia alter the human gut microbiome and induce intestinal barrier dysfunction and enhance the risk for infections (15). We previously reported that microbiome-derived metabolites can accumulate in breast tumors (16). Hence, we hypothesized that diabetes may influence tumor biology in breast cancer patients by mechanisms that may include the microbiome. Tumors in diabetic patients may acquire distinct molecular signatures that alter disease aggressiveness and therapy response.
To examine how diabetes affects breast cancer biology, we used a discovery approach consisting of three human xenograft models for breast cancer that were orthotopically grown in diabetes-prone NRG-Akita mice. We investigated the tumor metabolome and transcriptome and then compared the contrasts between hyperglycemic and control mice with the contrasts in human breast tumors comparing patients with diabetes to non-diabetic patients. In addition, we cultured human breast cancer cell lines under hyperglycemia for further discovery and performed mechanistic studies to validate observations. Using this approach, we identified coherent biological differences related to hyperglycemia and diabetes in both ER-negative and ER-positive breast cancer. Notably, our findings support the hypothesis that diabetes-associated breast tumors acquire a pro-inflammatory metabolome and a condition of DNA repair deficiency. Based on these observations, these tumors may show an increased response to DNA repair pathway inhibitors which should be examined in clinical studies.
RESULTS

Study design. The effects of diabetes and hyperglycemia on breast cancer biology have not been thoroughly investigated using clinical samples. Hyperglycemia is a hallmark of type I and type II diabetes whereas insulin secretion is reduced or absent when diabetes is established (Figure S1A-B)(17). We applied a three-pronged approach to obtain a comprehensive assessment of diabetes-induced effects in xenograft breast tumors, patient tumors, and human breast cancer cell lines as outlined in Figure S1C. Our animal model for diabetes/hyperglycemia was NRG-Akita mouse-based. We used female Akita mice that progressively develop hyperglycemia with an onset at 4 weeks of age as a model of genetically induced hyperglycemia with similarities to type 1 diabetes in disease origin but exhibiting some phenotypes of type 2 diabetes (18, 19). Fresh-frozen patient tumors were obtained from women with both type I diabetes (n = 6) and type II diabetes (n = 34). Most of these women were self-identified African Americans (n = 33), including all patients with type I diabetes. African American women are a high-risk group for aggressive forms of breast cancer and are generally more affected by diabetes than other women (6, 20).

Initially, we examined the hyperglycemia-induced biology of orthotopically grown tumors from 3 ER-negative human breast cancer cell lines, MDA-MB-231, MDA-MB-468, and Hs578T, injected into the abdominal mammary fat pad of either diabetes-prone Akita mice (NOD.Cg-Rag1 tm1Mom Ins2 Akita Ii2rg tm1Wj/SzJ) or its matched control (NOD.Cg-Rag1 tm1Mom Ii2rg tm1Wj/SzJ), all 8 weeks old. Tumor-bearing mice were sacrificed at 5 weeks for MDA-MB-231, 6 weeks for MDA-MB-468, or 8 weeks for Hs578T xenografts, as shown in Figure S2A. At these time points, all Akita mice, but none of the controls, had developed
hyperglycemia (n = 4-5 per group; Figure S2B). Xenografts in hyperglycemic and control mice did not show significant differences in either tumor growth (Figure S2C) or their proliferation score (Figure S2D) across the three models. Yet for the one cell line known to produce metastases from orthotopically grafted tumors, MDA-MB-231, we detected metastatic lesions in the spleen, kidney, and upper gastrointestinal tract in 2 out of 4 tumor-bearing Akita mice (50%) but not in any of the 5 tumor-bearing control mice (Figure S2E). Consistent with the xenograft growth data, hyperglycemia did not enhance proliferation in human breast cancer cells, irrespective if or not mannitol was added in the control experiments to adjust for osmolarity (Figure S2F).

**Hyperglycemia-induced metabolic alterations in breast tumor xenografts.** Next, we investigated the metabolome profiles of the xenografts comparing tumors from hyperglycemic versus control mice (n = 4, each comparison group). Being able to detect up to 830 metabolites with the applied platform (Metabolon), we uncovered hyperglycemia-associated alterations in the tumor metabolome (Table S1), as shown by the principal component analysis (PCA, including all metabolites, Figure 1A) and a hierarchical cluster analysis that included all metabolites at a false discovery rate (FDR) < 0.3 (MDA-MB-231, n = 219; MDA-MB-468, n =217; Hs578T, n = 443 metabolites; Figure 1B). These metabolic differences were also found in blood samples, with microbiome-derived 3-phenylpropionate and hippurate being the most up-regulated serum metabolites in presence of hyperglycemia, as shown for Akita mice bearing MDA-MB-468 xenografts (n = 4; Table S1; Figure S3A-D). Still, the hyperglycemia-associated metabolome contrasts for serum and tumor showed differences with 341 metabolites being distinctively altered by hyperglycemia in serum and 91 metabolites in tumor xenografts,
applying an FDR < 0.3 (Figure S4). Across the three xenograft models, 71 tumor metabolites were commonly altered in the Akita mice (Figure 1C; Table S2). Glucose was upregulated in all tumors of these mice, whereas intratumor 1,5-anhydroglucitol (1,5-AG), a known diabetes marker that is down-regulated in presence of hyperglycemia (21, 22), was greatly diminished (average 50-fold), consistent with diabetes/hyperglycemia-induced reprogramming of tumor metabolism (Figure 1D; Figure S3E-F). This observation was further confirmed with the concurrent accumulation of fructosyllysine (all xenografts) and N6-carboxylmethyllysine in MDA-MB-468 and Hs578T xenografts. Both metabolites belong to the family of food-derived, pro-inflammatory and pro-mutagenic advanced glycation end products that are commonly elevated in people with diabetes (23). Among the 71 tumor metabolites, 67 metabolites were either steadily increased (n = 53) or decreased (n =14) in Akita mice (Figure 1C), with an FDR < 0.05 for each metabolite in the combined analysis across the three xenograft models (Table S2). Notably, many of them represent food- or microbiome-derived metabolites that mostly accumulated in the tumors in presence of diabetes whereas a small number of metabolites represent typical energy or tumor metabolism-related molecules (e.g., α-ketoglutarate, glucose). Food-derived metabolites included isoflavones like genistein and daidzein sulfate that may reduce the risk of breast cancer recurrence but may also interfere with the antitumor effects of breast cancer therapeutics (24, 25). At least eight of the 71 diabetes/hyperglycemia-associated metabolites have previously been linked to the gut microbiome (26, 27) and included hippurate as the metabolite with the most significant accumulation, besides imidazole propionate, 3-phenylpropionate or phenyl sulfate, among others (Table S2). Hippurate, imidazole propionate, and phenyl sulfate have been reported to be increased in diabetes (26,
28, 29), consistent with our data. Lastly, we noticed that α-ketoglutarate, a key metabolite in the regulation and maintenance of the epigenome, was consistently down-regulated in tumors of hyperglycemic mice. Interestingly, a loss of α-ketoglutarate-dependent lysine demethylase activity has recently been linked to a suppression of DNA repair by disrupting local chromatin signaling (30, 31).

**The metabolome of diabetes-associated patient tumors.** Having observed that diabetes-related hyperglycemia alters the metabolome of human breast tumor xenografts, we asked whether similar metabolic alterations can be detected in breast tumors from patients with diabetes. We analyzed the metabolome of tumors from 40 women with diabetes and 48 without diabetes and used the tumor ER-status to match patients (Table S3). Most of these patients were self-identified African American women. Not unexpected, patients with diabetes were older (65 vs. 51.5 years of age) and tended to have a higher body-mass index (BMI) (32.2 vs. 29.2). Even so, independent of the diabetes status, most women in our patient cohort would best be categorized as overweight to obese, representing US trends for women in this age group. The metabolome contrast comparing diabetic with non-diabetic patients was consistent with the metabolome contrast in our experimental model of hyperglycemia, however less distinct likely because patients were heterogenous with regards to their dietary intake and being treated with anti-diabetic drugs. Nevertheless, when we choose an unadjusted $P < 0.05$ as the cutoff, a metabolome profile emerged that was consistent with findings in our xenograft-based discovery cohort (Figure S3G-I). Diabetic patients presented with reduced intratumor 1,5-AG levels and an accumulation of microbiome-derived metabolites in their tumors, including trimethylamine N-oxide, imidazole propionate, cresol sulfate, and phenyl sulfate.
Food-derived metabolites included the advanced glycation end product, N6-carboxymethyllysine (CML). The intratumor accumulation of CML was robust and remained significant after further adjustments for age, race, BMI, tumor stage, and tumor ER status. CML also accumulated in the tumor xenografts and has been described as a candidate ligand of the RAGE receptor that has candidate oncogenic and pro-inflammatory functions in cancer (32). We examined if CML at the physiological concentration of 1 µM (33) would induce a proinflammatory RAGE signaling signature in MDA-MB-231 breast cancer cells. As shown in Figure S5A-C, CML induced such a signature with increased NFκB-mediated TNFα signaling as the top-ranked pathway. Other activated pathways included Myc and TGFβ signaling, upregulation of reactive oxygen species (ROS), the inflammatory response, and epithelial-to-mesenchymal transition (EMT).

**Diabetes-induced transcriptome is consistent with the activation of developmental pathways and a decreased DNA repair capacity.** To continue our interrogation of the diabetes-associated tumor biology, we generated RNA sequencing-based gene expression profiles for the 3 tumor xenograft models, 73 patient tumors, and 4 human breast cancer cell lines from African American patients cultured under hyperglycemic conditions (ER-positive: MDA-MB-175, ZR-7530, and HCC1500, ER-negative: MDA-MB-157, MDA-MB-231 and MDA-MB-468). The 73 patient tumors were obtained from 36 patients with and 37 without diabetes and represent a subset of the tumors with metabolome data (Table S3). Comparing the gene expression profiles between tumors from diabetic versus non-diabetic patients revealed large differences with 2012 differentially expressed genes at an FDR < 0.05 (Table S4). A hierarchical cluster analysis using the 673 differential genes at an FDR < 0.05, with additional covariate adjustments for age,
race, BMI, tumor stage, and tumor ER status in generating the contrast, achieved a separation into tumors from diabetic and non-diabetic patients (Figure 2A), indicating that the impact of diabetes on tumor biology is well captured by the tumor transcriptome. This finding was replicated in xenografts and cultured cells. In Akita mice, experimental diabetes induced robust gene expression signatures in MDA-MB-231 and MDA-MB-468, but not Hs578T, tumor xenografts (Figure S6A-E). Similarly, the gene expression profiles of breast cancer cell lines cultured under hyperglycemia showed robust changes (Figure S6F-M).

Next, we applied gene set enrichment analysis (GSEA)(34) using the Hallmark and KEGG pathway gene set collections to initially interrogate patient tumors (Figure 2B-C; Table S5). The examination was performed with the covariate-adjusted gene list for the contrast diabetic versus non-diabetic (covariates: age, BMI, race, disease stage, tumor ER status) and revealed diabetes-associated gene signatures that included the induction of a hedgehog- and myogenesis-related gene expression program, and increased notch and EMT signaling among the top-ranked pathways, pointing to a common activation of developmental and oncogenic pathways in tumors of people with diabetes (all FDR < 0.25). Myogenesis is critical to muscle development and has close links to hedgehog signaling. Hallmark myogenesis has recently been linked to a high-risk breast cancer subtype of increased mobility (35). Myogenesis and hedgehog signaling were found to associate with poor prognosis in breast cancer by these authors (35). We followed up on this finding with an analysis using single sample GSEA (ssGSEA)-based pathway scores (see supplemental methods) that capture pathway activation in individual breast tumors. With this approach, we could further demonstrate that myogenesis and hedgehog signaling are up-regulated in breast tumors from diabetic patients largely
independent of the tumor ER status (Figure 3A-F). We also found that the myogenesis-related gene expression program was induced by diabetes and hyperglycemia in all human tumor xenografts and breast cancer cell lines, among the top-ranked pathways, whereas the hyperglycemia-induced EMT signature was most notable in the ER-positive cell lines (Figures S7A-G; Table S6). Inflammation-related pathways, including increased TNFα signaling, were commonly activated in both tumor xenografts and breast cancer cell lines when exposed to hyperglycemia.

GSEA Hallmark pathway enrichment scores revealed that the DNA repair capacity might be reduced in human breast tumors from diabetic patients (Figure 2B). We followed up on this observation using GSEA KEGG pathway assignments that better define DNA repair pathways (Figure 2C) and generated pathway activity scores for individual tumors. This line of inquiry provided further indication of a broadly reduced DNA repair capacity in patient tumors (Figure 3G-K) and human xenografts (Figure S8A, Table S7). Down-regulation of the repair pathways in patient tumors occurred independent of the tumor ER status (Figure S8B-C). Most prominent was the deficiency in KEGG pathway-annotated base excision repair, mismatch repair, and homologous recombination. We observed a similar down regulation of DNA repair pathways in breast cancer cell lines (Table S7). A homologous recombination deficiency typically associates with “BRCAness” of breast tumors due to mutational inactivation of the BRCA1 and BRCA2 tumor suppressor genes, making these tumors susceptible to poly-ADP ribose polymerase inhibitors. Yet, diabetes may induce “BRCAness” by an alternative mechanism involving loss of gene expression as BRCA1 and BRCA2 transcripts were commonly downregulated in the clinical samples and xenografts in presence of diabetes (Figure S9A-B).
Finally, we performed an integration of transcriptome and metabolome data to identify candidate pathways that may have a key function in defining the effects of diabetes on tumor metabolism. To do so, we combined the transcriptome and metabolome data from across the 24 breast tumor xenografts using first a correlation analysis followed by GSEA with genes ranked by their correlation coefficient (see methods). We restricted this analysis to correlations of gene expression with the 67 metabolites that were consistently increased (n = 53) or decreased (n = 14) in tumors of diabetic Akita mice (see Table S2). This exploratory approach showed that myogenesis and mitochondrial oxidative phosphorylation as the top ranked pathways may mediate the effect of diabetes on tumor metabolism in these xenografts (Table S8).

**Diabetes and hyperglycemia induce an invasive phenotype.** Diabetes did not significantly influence tumor xenograft growth (Figure S2C-D) but may promote metastasis, as shown for the implanted MDA-MB-231 cells (Figure S2E, Figure S10A). Thus, we assessed the effect of diabetes on both cell proliferation and a pro-metastatic phenotype in patient tumors using the transcriptome data and examined how hyperglycemia may affect human breast cancer cells in culture. Tumors from diabetic patients exhibited decreased proliferation in both ER-negative and ER-positive tumors (Figure 4A-C), as judged by their proliferation score derived from a validated gene expression proliferation signature (16, 36). In contrast, the Hallmark EMT signature was upregulated in breast tumors of patients with diabetes (Figure 4D-F). Transcript levels of several EMT driver genes, e.g., ZEB1, VIM, TWIST1, were elevated in breast tumors of patients with diabetes (Figure S11) and the diabetes-associated transcriptome showed significant overlap with the Hollern_EMT_Breast_Tumor_Up as well as a LIM-
Mammary Stem Cell Up signatures in the GSEA MSigDB collection (Figure 4G-H; FDRs = 0.1 and 0.06, respectively). To corroborate that hyperglycemia induces a mesenchymal phenotype with increased mobility, we measured migration and invasion under hyperglycemic conditions of human breast cancer cells and also determined the acquisition of a mesenchymal phenotype by quantifying cell length in culture to capture a spindle-like morphology. In agreement with the pathway analysis, hyperglycemia increased cell migration (Figure 5A-C), but not proliferation (Figure S2F), and induced a more spindle cell-like appearance as quantified by an increased average cell length (Figure 5D-G). Additionally, we performed the Matrigel invasion assay, where we observed that hyperglycemia increased breast cancer cell invasion (Figure 5H-I). To further validate that hyperglycemia may increase stemness in breast cancer cells, we cultured MDA-MB-231-LM2 cells carrying a reporter construct in which six concatenated repeats of a composite SOX2/OCT4 response element from the proximal human NANOG promoter are coupled to a minimal cytomegalovirus (CMV) promoter, to drive expression of a fluorescent reporter gene for stem cell signaling (37). Using this cell line, we show that our standard hyperglycemic culture conditions induce the fluorescent reporter, thereby showing increased stemness (Figure 5J). Finally, transcript levels of multiple cancer stem cell markers tended to be upregulated in breast tumors of patients with diabetes (Figure S12).

**Hyperglycemia induces mitochondrial oxyradical formation and DNA damage and increases sensitivity to DNA repair inhibitor drugs.** Oxidative stress due to hyperglycemia has been linked to lung metastasis in a syngeneic mouse metastasis model (38). Hence, we asked if hyperglycemia may cause oxidative stress and increases DNA damage in human breast cancer cells. We confirmed the elevated production of mitochondrial ROS by fluorescence activated...
cell sorting (Figure 6A-D), albeit with an estimated moderate increase, and found the ROS scavenger Mitotempo to inhibit hyperglycemia-induced cell migration, indicating that upregulation of ROS production is an inducer of migration under hyperglycemia in these cells (Figure 6E-H). The observation is in agreement with the literature showing that moderate rather than sizable increases of ROS enhance metastasis (39). To examine whether hyperglycemia and ROS may increase DNA damage, we examined the number of cells positive for the two DNA damage markers, γH2AX (40) and 53BP1 (41), using immunofluorescence microscopy. Both markers were significantly elevated in Hs578T and MDA-MB-231 cells when cultured under hyperglycemia (Figure 7A-D, Figure S9C-D). In addition to nuclear staining, we also observed diffuse cytoplasmic staining of γH2AX in presence of hyperglycemia. A previous report suggested a DNA repair deficiency can lead to accumulation of fragments of unrepaired genomic DNA in the cytoplasm, with increased cytoplasmic γH2AX (42). This notion of increased DNA damage and a reduced DNA damage repair capacity was further supported by an Ingenuity Pathway Analysis (IPA) that included 461 differentially expressed genes across patient and xenograft tumors (diabetic versus non-diabetic; Table S9) showing that “the role of BRCA1 in DNA damage response” is the top down-regulated IPA-defined process in presence of diabetes (Figure 8A-B). The downregulation of these DNA repair pathways was also suggested when we performed another IPA analysis with the 311 genes whose expression was similarly altered by hyperglycemia (95 up-regulated and 216 down-regulated, FDR < 0.3) across the MDA-MB-231 and MDA-MB-468 xenografts and their corresponding cell lines (Figure S13A-B, Table S9).

Further, using a reporter assay to measure non-homologous end joining (NHEJ) DNA repair capacity, we could demonstrate a decrease in NHEJ in MDA-MB-231 and Hs578T breast
cancer cells when cultured under hyperglycemia (Figure 9A-D). A downregulation of this pathway activity is consistent with partial BRCA1/2 inhibition in presence of diabetes and hyperglycemia (Figure S9A-B).

We followed up on our observations with an analysis of nuclear γH2AX positivity in 105 breast tumors from 29 patients with diabetes and 76 patients who did not have a diagnosis of diabetes. γH2AX positivity in the tumor epithelium was present in 58.6% of the diabetic patient (17/29) and 36.8% of the non-diabetic patients (28/76) (P = 0.05, two-tailed Fisher’s exact test). We obtained a moderate to high γH2AX immunostaining score for 44.8% of the tumors from patients with diabetes (13/29), whereas 30.2% of the tumors from non-diabetic patients (23/76) scored in this range (P = 0.17).

Because pathway analysis pointed to a broadly reduced DNA repair capacity in breast tumors of patients with diabetes, we treated multiple breast cancer cell lines (Hs578T, MDA-MB-468, MDA-MB-231, MDA-MB-436 and HCC1937) with drugs targeting DNA repair mechanisms, namely AZD7762, berzosertib, etoposide, and olaparib, and examined drug sensitivity in a BrdU incorporation-based cell proliferation assay (Figure 10). For AZD7762, berzosertib, and etoposide, we determined the IC50 under the control condition (5 mM glucose) and hyperglycemia (25 mM glucose), whereas for olaparib, we determined BrdU incorporation for the 1, 10, and 20 µM concentration range as the IC50 tended to exceed 50 µM, in agreement with the literature (43). MDA-MB-436 and HCC1937 are cell lines that harbor mutant BRCA1 (44). The five cell lines showed a generally increased sensitivity to these drugs under hyperglycemia, as defined by their decreased IC50 drug response values (Figure 10). For olaparib, the two BRCA1 mutant cell lines had the weakest differential response under the two
culture conditions. Similarly, when using a CellTiter blue-based viability assay and a defined drug concentration with 4 cell lines, we observed a decrease in viability by about 20-30% under hyperglycemia when compared to control cells at the 48 hours exposure timepoint (Figure S14).

**Diabetes associates with a distinct tumor mutational signature in breast cancer patients.**

Because hyperglycemia increased DNA damage in cultured cells, we asked whether breast tumors from patients with diabetes may acquire an increased mutational burden or a distinct mutational signature. Using whole exome sequencing, we analyzed 38 breast tumors from diabetic patients, 71 tumors from patients without diabetes, and 7 tumors from patients who developed diabetes on follow-up. We did not find obvious differences in the overall frequency of somatic mutations comparing tumors by patients’ diabetes status (Figure 11A, Table S10). However, we found that tumors from patients with diabetes contain signatures of a reduced DNA damage repair capacity (SBS5 and SBS30) related to base excision repair (Table S10). In an analysis that applied subject/signature age-adjusted weights to control for age differences between patients with and without diabetes (45) and comparing individual samples versus reference signatures from the COSMIC catalog (46, 47) and the Compendium of Mutational Signatures of Environmental Agents (48), we could identify several signatures with a disparate prevalence among the non-diabetes and diabetes groups (two-sided Wilcoxon test, $P < 0.05$ in the unadjusted analysis) (Figure 11B, Table S10). Of those two COSMIC signatures, namely SBS5 ($P = 0.001; \text{FDR} = 0.057$) and SBS30 ($P = 0.007; \text{FDR} = 0.187$), stood out with being most robustly upregulated in tumors of patients with diabetes. The frequencies of these two signatures were similarly elevated in ER-positive (SBS5, $P = 0.026$; SBS30, $P = 0.031$) and ER-negative tumor (SBS5, $P = 0.043$; SBS30, $P = 0.024$). Notably, both signatures are associated with impaired DNA
repair. SBS5 is increased in bladder tumors with mutations in the DNA excision repair gene ERCC2 whereas signature SBS30 relates to a deficiency in base excision repair due to inactivating mutations in NTHL1. Enrichment of these signatures in association with diabetes is consistent with the impairment of DNA damage repair pathways that was predicted from the transcriptome analysis. In a last analysis, we compared the fraction of mutated samples for genes with a mutation in more than 5% of the samples (Figure 12, Table S10). We found that several genes, including TP53, PIK3CA, MUC5AC, and CDH1, had higher mutation frequencies in tumors of diabetic than non-diabetic patients, with mutation frequencies for TP53, PIK3CA, MUC5AC and CDH1 being 37%, 21%, 16%, and 11%, respectively, in diabetic patients but 25%, 13%, 4%, and 4% in non-diabetic patients. However, these differences did not reach statistical significance (Table S10). We did not find that mutations in BRCA1/2 or other repair genes are increased in breast tumors of diabetic patients, suggesting that loss of gene expression rather than mutational inactivation is the underlying mechanism of the DNA repair deficiency in these tumors.
DISCUSSION

Diabetes commonly affects breast cancer patients and 1 out of 6-8 women with breast cancer has diabetes as a comorbidity (4, 49). It affects African American women with breast cancer more so than European American women (6, 50). In people with diabetes, breast cancer tends to have more aggressive features and to associate with decreased patient survival (4, 6, 51). Despite the evidence that diabetes may affect disease outcome and response to therapy, we are still lacking an understanding of the diabetes-induced molecular changes in human breast tumors.

Here, we used a broad approach that included analysis of patient tumors, orthotopic human tumor xenografts, and human breast cancer cells exposed to diabetes and hyperglycemia to gain an understanding how diabetes may alter the tumor biology in breast cancer patients. We found that food- and microbiome-derived metabolites accumulate in breast tumors in presence of diabetes and hyperglycemia. In contrast, α-ketoglutarate is consistently down-regulated in tumors of hyperglycemic mice, which may lead to a partial inhibition of ketoglutarate-dependent enzyme activities. Importantly, the loss of α-ketoglutarate-dependent lysine demethylase activity, namely of KDM4B, has recently been linked to a suppression of DNA repair by disrupting local chromatin signaling (30, 31).

Diabetes also induced EMT- and stem cell-like phenotypes generally linked to dedifferentiation, increased mobility, and odds of metastasis. Furthermore, and perhaps most significant, diabetes associated with gene expression and mutational signatures of a DNA damage repair deficiency. Most of these phenotypes occurred in both ER-negative and ER-positive breast tumors. The repair deficiency may partly relate to a diabetes-associated down-
regulation of BRCA1/2 function in breast tumors, as our data suggest, and to metabolic alterations that increase oxidative stress and reduce the availability of α-ketoglutarate. Still, other repair pathways that are not dependent of BRCA1/2 function were similarly inhibited. Correspondingly, breast cancer cells cultured under hyperglycemia acquired increased DNA damage and sensitivity to DNA damage response inhibitors. Hence, diabetes-associated breast tumors may show an augmented drug response to DNA damage repair pathway inhibitors that are cancer therapeutics.

The effect of diabetes and hyperglycemia on tumor growth and metastasis has been studied in syngeneic mouse tumor models including the 4T1 and E0771 breast cancer models (12-14, 38). Some of these studies observed an increased tumor growth induced by diabetes (13, 14) whereas others did not (12, 38). We did not find any evidence of a growth accelerating effect by diabetes/hyperglycemia in human breast cancer cells. In patient tumors, diabetes associated with a reduced proliferation score in both the ER-negative and ER-positive disease. Yet, consistent with the mouse tumor model, we obtained evidence of a diabetes-induced mesenchymal transition, increased migration, and increased odds of metastasis in \textit{in vitro} and \textit{in vivo} models of human breast cancer. Also consistent with the literature (38, 52), hyperglycemia increased mitochondrial ROS production in human breast cancer cells, with ROS being a key factor in the increased mobility of these cells. Having made these observations, one would argue that diabetes increases the odds of metastasis in humans rather than breast cancer growth. Nonetheless, there is evidence from epidemiology that diabetes not only increases mortality of breast cancer patients (4, 6, 51) but also raises the disease risk (53).
Myogenesis is the formation of skeletal muscular tissue during embryonic development and is important in muscle tissue regeneration. The latter is commonly inhibited in cancer patients and leads to the condition of cachexia with extreme loss of skeletal muscle tissue (54, 55). The upregulation and oncogenic role of myogenic transcription factors (MYF5, MYOD) has been described for rhabdomyosarcoma (56), a pediatric malignancy of muscle. To our understanding, myogenesis has not been recognized as an oncogenic signaling pathway that functions in epithelial cancers. However, upregulated myogenesis has recently been linked to a high-risk breast cancer subtype of increased mobility (35). In our study, myogenesis was consistently identified as the top-ranked biological process that is activated in both ER-negative and ER-positive breast tumors of patients with diabetes compared to non-diabetic patients, and in breast tumor xenografts and breast cancer cell line under hyperglycemia. As Hallmark myogenesis encompasses hedgehog and notch signaling, the upregulation of this biological process by diabetes may broadly reflect the activation of developmental pathways that become oncogenic in the context of cancer and promote increased cell mobility.

We investigated the diabetes-associated metabolome using untargeted metabolomics and measured 830 metabolites, including the diabetes marker, 1,5-AG, in the tumor tissues which is a strength of our study design. The analysis of our human xenograft data showed that diabetes and hyperglycemia influence the tumor metabolome, leading to the accumulation of food- and microbiome-derived metabolites in tumors as a key feature of diabetes-induced alteration under controlled conditions. We did not find the same robust changes in breast cancer patients, likely because of the heterogeneity in their diet/lifestyle and management of diabetes, something one cannot easily control in a patient population. Nevertheless, diabetic
patients presented with reduced intratumor 1,5-AG levels and an increase in food- and microbiome-derived metabolites, both consistent with the breast cancer xenograft data. Reduced 1,5-AG is a biomarker of glucose spikes and has been found to associate with a generally increased cancer mortality in a study of Japanese men (22). The accumulation of microbiome-derived metabolites that we observed in the tumor xenografts may contribute to the proinflammatory environment with increased ROS that has been described from syngeneic breast tumors in diabetic mice; however, none of these previous studies investigated the tumor metabolome that associates with diabetes. Several diabetes-associated metabolites, e.g., imidazole propionate, trimethylamine N-oxide, and phenyl sulfate, are formed by the gut microbiome and may render cells to increased oxidative stress and the tumors to inflammation (28, 29, 57-59).

Advanced glycation end products are sugar metabolites that build up in patients with diabetes. They are pro-inflammatory and mutagenic and oncogenic, and their accumulation may raise breast cancer risk and mortality (32, 60-62). We detected an increased intratumor abundance of them under diabetic conditions and could show that one of the best-known advanced glycogen end products, CML, activates oncogenic and inflammatory pathways in breast cancer cells, in line with RAGE (receptor for advanced glycation end products) signaling. Thus, besides microbiome-derived metabolites, the increased exposure to advanced glycation end products may as well contribute to the more aggressive nature of breast cancer in diabetic patients.

Oxidative stress promotes DNA strand breaks and genomic instability in cancer cells. Hyperglycemia increases ROS and DNA damage, as shown by our data and by others (38). At the
same time, these cells may also experience a loss in DNA damage repair capacity. A compromised DNA repair capacity has previously been linked to diabetes-induced fibrosis (63). We found that a deficiency in base excision repair, mismatch repair, and homologous recombination is strongly suggested by the gene expression profile and mutational signature in breast tumors from patients with diabetes, independent of the tumor ER status. Two mutational signatures that were present in these tumors may originate from a reduced DNA excision repair capacity, as suggested by the COSMIC signature compendium. Our observations from patient tumors were further confirmed by the transcriptome data obtained from our mouse xenograft model using diabetes-prone Akita mice and experimental data from high glucose-cultured human breast cancer cell lines.

Homologous recombination is a key pathway in repairing double strand breaks that frequently occur under oxidative stress (64). Homologous recombination deficiency due to mutational inactivation of the \textit{BRCA1} and \textit{BRCA2} tumor suppressor genes is known to make human tumors susceptible to poly-ADP ribose polymerase (PARP) inhibitors and platinum-based chemotherapies (65). PARP inhibitors have therefore been developed to treat cancer patients with \textit{BRCA} inactivating mutations (66). We investigated if human breast cancer cells when cultured under hyperglycemia are more vulnerable to DNA repair inhibitors, assessing a panel of cancer drugs that included the checkpoint kinase inhibitor, AZD7762, the ATR (ataxia telangiectasia and Rad3 related) inhibitor, berzosertib, the topoisomerase II inhibitor, etoposide, and the PARP inhibitor, olaparib. This line of experiments indicated a raised vulnerability of human breast cancer cells to these drugs under hyperglycemic conditions. We also showed that hyperglycemia down-regulates DNA repair capacity, namely non-homologous
end joining, which is the primary pathway for repair of double stranded breaks throughout the cell cycle including the G2 phase, using a reporter assay. Based on these observations, we reason that diabetes-associated breast tumors may show an increased drug response to DNA damage repair inhibitors which are cancer therapeutics. We further argue that these observations should be followed up with clinical studies. Diabetes negatively and disproportionately impacts underserved populations (20) and increases breast cancer mortality on a global scale. Any improvement in treating these breast cancer patients should make a large impact.

As a strength of our study, we used a comprehensive approach to investigate the effects of diabetes and hyperglycemia on breast cancer biology and included tumors and cell lines from African American breast cancer patients. However, breast cancer patients with diabetes will use a variety of drugs that treat diabetes. Their use should make diabetic patients more similar to non-diabetic patients, thus dilute the signal of diabetes. Nevertheless, it is a limitation of our study that we could not investigate how treatment of diabetes may have influenced our tumor data. We also could not associate duration of diabetes and circulating hemoglobin A1c levels with our tumor data as these data were only available for a subset of the patients in our study. Another limitation of our study relates to the use of the Akita mouse as a diabetes model. The disease resembles type 1 diabetes but shows some phenotypes of type 2 diabetes (18, 19). These mice are immune compromised but develop hyperglycemia with 100% penetrance in a well-defined age range allowing tumor xenografts to grow in mice with diabetes with little experimental variation. It has been shown that hyperglycemia leads to immune function changes in breast tumors using syngeneic mouse models (14). Despite these limitations, the
transcriptome- and metabolome-based signatures in the human xenografts grown in diabetic Akita mice showed significant overlaps with the signatures detected in breast tumors of diabetic patients. They point to the same consistent alterations across these lines of investigation, pointing to identical diabetes/hyperglycemia-induced effects in them. These findings make us confident that our observations are valid, thereby supporting our study design. In addition, the key findings that hyperglycemia induces ROS and a condition of DNA repair deficiency were experimentally validated.

In summary, our investigation reveals an impact of diabetes on the biology of human breast tumors, largely independent of the tumor ER status, and provides a large dataset of metabolome and transcriptome data as a rich resource for others to examine the effects of diabetes in patient tumors. Diabetes may broadly activate developmental pathways that become oncogenic in the context of cancer and promote increased cell mobility and the odds of metastasis. At the same time, food- and microbiome-derived metabolite increase in these breast tumors, potentially affecting tumor biology by increasing diabetes-associated inflammation. Through increase of ROS, diabetes also augments DNA damage in cancer cells while diminishing their abilities of repairing DNA lesions and strand breaks. Clinically, these events may lead to an increased drug response to DNA damage repair inhibitors among patients with diabetes-associated breast cancer.
METHODS

Reagents. All information is provided in supplemental methods.

Collection of human breast tumors and patient data. Breast cancer patients undergoing surgery were recruited at the University of Maryland Medical Center. We previously described recruitment of this patient cohort (16, 67). Details are provided in supplemental methods.

Orthotopic tumor growth in mice with diabetes/hyperglycemia. Female Akita mice (NOD.Cg-Rag1 tm1Mom Ins2 Akita Il2rg tm1Wjl/SzJ) (68) were obtained from Jackson Laboratory (Bar Harbor, ME). This mouse strain is partly immunodeficient and develops hyperglycemia with a defined onset at 4 weeks of age. The gut microbiome is intact and contains the typical microbiome of JAX mice (personal communication, Jackson Laboratory In Vivo Services). The animals are heterozygote carriers of a mutation in the insulin 2 gene. The mutation induces misfolded protein and β cell death. Akita mice are a model of genetically induced hyperglycemia with similarities to type 1 diabetes but exhibit some phenotypes of type 2 diabetes (18, 19). We used the isogenic strain, NOD.Cg-Rag1 tm1Mom Il2rg tm1Wjl/SzJ (69), as a matched control in this study. Further information and description of the orthotopic xenograft experimental protocol can be found in the supplemental methods.

Cell lines. All human breast cancer lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA). MDA-MB-157, MDA-MB-231, MDA-MB-436, and HCC1806 cells were grown in DMEM media with 2 mM glutamine (Millipore Sigma) and 10% FBS. MDA-MB-175, MDA-MB-468, Hs578T, ZR-75-30, HCC1937, and HCC1500 cells were grown in RPMI media supplemented with 10% FBS. We obtained authentication of these cell lines through ATCC services, prior and after completion of all experiments, using a short tandem repeat analysis.
MDA-MB-231-LM2 mCMV-mcherry and MDA-MB-231-LM2-SORE6-mcherry cell lines were obtained from Lalage M. Wakefield (NCI, Bethesda, MD). These cell lines were maintained in RPMI media supplemented with 10% FBS.

**Hyperglycemia in cell culture.** We performed *in vitro* experiments mimicking diabetes-like condition by culturing breast cancer cells in high glucose medium. We used 5 mM glucose level as control condition and 25 mM levels to model hyperglycemia (70). Both ER-positive (MDA-MB-175, ZR-75-30, HCC1500) and ER-negative (MDA-MB-157, MDA-MB-231, MDA-MB-468, Hs578T) human breast cancer cell lines were used for these experiments. Briefly, cells were plated in respective plates/flasks. The next day, the medium was removed, and cells were washed with PBS and then cultured with either low (5 mM) or high glucose (25 mM) media without serum. Prior to starting the high glucose treatment experiments, we conditioned the cells with low glucose medium, then switched to 25 mM glucose while we maintained the 5 mM glucose for the control cells. In some experiments, we added 20 mM mannitol to the 5 mM glucose control group to yield the osmolarity of added 25 mM glucose. However, we did not find differences in the investigated phenotypes between experiments with and without added 20 mM mannitol (*Figure S2F-G*).

**Bromodeoxyuridine (BrdU) incorporation assay to assess inhibition of cell proliferation by DNA repair pathway inhibitors.** Cell proliferation was measured with the BrdU cell proliferation kit from Millipore Sigma. For details see supplemental methods.

**Cell viability assay.** Numbers of viable cells were estimated using the CellTiter-Blue® cell viability assay from Promega. For details see supplemental methods.
**Migration and invasion assay.** Cell migration and invasion was monitored using the xCelligence System technology (Roche Applied Science/ACEA Biosciences, Inc, San Diego, CA) for real-time monitoring of cell movement with an electronic cell sensor array, following manufacturer’s instructions. For more details see supplemental methods.

**Measurement of mitochondrial ROS.** Mitochondrial superoxide in the breast cancer cells was measured following an earlier described method (71). For details see supplementary methods.

**Immunofluorescence microscopy to quantify DNA damage.** Immunostaining of DNA damage markers (gamma H2A.X and 53BP1) was performed in MDA-MB-231 and Hs578T cells under hyperglycemic condition. Procedures are described in the supplemental methods.

**γH2AX immunohistochemistry (IHC).** Accumulation of nuclear γH2AX protein was examined in 105 formalin-fixed and paraffin-embedded breast tumor tissues obtained from the Department of Pathology at the University of Maryland Clinical Center. Twenty-nine tumors [17 ER-positive (59%), 11 ER-negative (38%), 1 ER unknown] were obtained from patients with diabetes at time of disease diagnosis and 76 [45 ER-positive (59%), 30 ER-negative (39%), 1 ER unknown] from patients who were non-diabetic at disease diagnosis. We used the anti-phospho-histone H2A.X (Ser139) rabbit antibody from Cell Signaling (catalog # 9718) at a 1:800 dilution to visualize γH2AX protein in the tumor sections. Nuclear γH2AX in the tumor epithelium was scored as negative, low, moderate, or high using a standard scoring system as previously described by us and others (72, 73). Scoring of the immunohistochemistry was performed by a pathologist blinded to the diabetes status of the patients.

**Measurement of DNA repair capacity for non-homologous end joining (NHEJ) in breast cancer cells under hyperglycemic condition.** Assessment of the DNA repair capacity for NHEJ was carried out according to a previously described protocol (74), with some modifications. For details see supplemental methods.
Quantification of the mesenchymal phenotype in cell culture.

The quantification of mesenchymal morphology in Hs578T and MDA-MB-231 cells cultured under hyperglycemia is described in supplemental methods.

**Stemness reporter assay.** The assay has previously been described (37). For details see supplemental methods.

**IC50/GI50 calculation using the GraphPad software.** Dose response measurements were analyzed using the GraphPad Prism software. The IC50 (alternatively defined as GI50 – as the concentration that results in inhibiting cell growth by 50%) for the various DNA repair pathway drugs under low and high glucose culture condition was calculated using nonlinear regression analysis (Fitting a dose-response curve) in GraphPad prism 9. First, 100% (average absorbance of the control group) and “0% growth” (average absorbance blank from media without cell) were assigned in the growth curve. Then, the doses were log transformed and absorbance values were normalized. The IC50 was calculated by nonlinear regression analysis (curve fitting) of normalized transformed data by selecting the model “absolute IC50 from normalized data”.

**Tumor proliferation score.** We selected a gene expression profile that included expression data for 11 cell-cycle genes (**BIRC5**, **CCNB1**, **CDC20**, **CEP55**, **MKI67**, **NDC80**, **NUF2**, **PTTG1**, **RRM2**, **TYMS**, **UBE2C**) and summed this profile into a meta-gene score as a marker for tissue proliferation, as described previously (16). The proliferation signature also contains **MKI67**, the transcript that encodes Ki67, a commonly used proliferation marker for tissues.

**Metabolome analysis of human tumors and xenografts.** The metabolome analysis of fresh-frozen human tumors and xenografts were performed using untargeted metabolic profiling by the service provider, Metabolon, Inc. (Morrisville, NC). For details see supplemental methods.
Transcriptome analysis of human tumors, xenografts, and cultured cells using RNA-sequencing. RNA was isolated from frozen breast tumors with/without diabetes (n = 36 diabetic and n = 37 non-diabetic) using the TRIzol method as described earlier (67). Isolation of RNA from the human xenografts (MDA-MB-231, MDA-MB-468 and HS578T +/- diabetes), breast cancer cells (MDA-MB-175, ZR7530, HCC1500, MDA-MB-157, MDA-MB-231 and MDA-MB-468) cultured under high glucose (25 mM), and MDA-MB-231 cells ± CML was done using the RNeasy Plus Mini Kit. Detailed information about the RNA sequencing methods, data analysis, and public access to the data is provided with the supplemental methods.

Metabolomic and transcriptomic data integration. For details see supplementary methods.

Whole exome sequencing (WES). WES was performed by the service provider, Psomagen (Rockville, MD). Detailed information about the WES data generation, data analysis, and public access to the data is provided with the supplemental methods.

Gene Set Enrichment Analysis (GSEA) and further validation using pathway “activity” scores. GSEA and additional validation of the key pathways using activity scores are described in the supplemental methods.

Single Sample Gene Set Enrichment Analysis (ssGSEA). For details see supplementary methods.

IPA Pathway analysis. Genes commonly up- and down-regulated in human breast tumors and xenografts (MDA-MB-231 and MDA-MB-468) comparing diabetic versus non-diabetic were uploaded into the IPA tool (n = 461 at FDR < 0.3 for differentially expressed genes) to analyze for their relationship with IPA pathways by calculating pathway enrichment scores. IPA maintains a large-scale pathway network derived from the Ingenuity Knowledge Base, which is a large, structured collection of observations in various experimental contexts with nearly 5
million findings manually curated from the biomedical literature or integrated from third-party databases. IPA calculates an enrichment score and \( P \) values using the Fishers exact test.

**Statistical analysis.** All statistical tests were 2-sided, and an association was considered statistically significant at \( P < 0.05 \). Statistical analyses were performed using either Prism 8 (GraphPad), the R software (https://www.r-project.org), the packages in Bioconductor provided by the R Foundation for Statistical Computing, or with Qlucore Omics Explorer 3.7 (https://qlucore.com/omics-explorer).

**Study Approval.** Collection of both biospecimens and the clinical and pathologic information was approved by the University of Maryland Institutional Review Board (UMD protocol no. 0298229). The research was also reviewed and approved by the NIH Office of Human Subjects Research Protections (OHSRP no. 2248). Informed written consent was obtained from all patients and the research followed the ethical guidelines set by the Declaration of Helsinki.

**Data Availability.** Metabolome data were deposited in the Open Science Framework (https://osf.io) at https://osf.io/h73rf/?view_only=. All RNAseq data generated for this study have been deposited in NCBI’s Gene Expression Omnibus (GEO) database under the super series GSE202923, which is composed of subseries GSE202922, GSE202599, GSE202595, GSE202597, GSE202598 and GSE236420. The RNA-Seq data for the human breast tumors were deposited under accession number GSE202922. The RNA-Seq data for the mouse xenografts were deposited under accession number GSE202599. RNA-Seq data for the cell lines were deposited under accession numbers GSE202595 (ER-positive cell lines), GSE202597 (CML-exposed MDA-MB-231 cells), GSE202598 (MDA-MB-157 cell line) and GSE236420 (MDA-MB-
The WES raw data for the breast tumors have been deposited in the SRA database under accession number PRJNA840859.

All data values referred to in the main manuscript and supplemental materials including the values for all data points shown in graphs and the values to support any reported means are provided in a single Excel (XLS) file. This excel file has been uploaded as “Supporting data values” with this manuscript.

Author contributions


Acknowledgments

We would like to thank personnel at the University of Maryland and the Baltimore Veterans Administration Hospital for their contributions with the recruitment of participants into the NCI-Maryland breast cancer study. This work was supported by the Intramural Research Program of the NIH, National Cancer Institute (NCI), Center for Cancer Research (ZIA BC 010887
to S.A.) and National Institute of Minority Health and Health Disparity. We thank Richa Maheshwari and Orna Cohen-Fix at National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) for their assistance in confocal microscopy. We thank Binwu Tang and Lalage M. Wakefield at NCI for providing the MDA-MB-231-LM2 cell line carrying the SORE6 reporter construct.

Declaration of interests

The authors declare that they have no competing interests.

Supplemental Information

Supplemental Figures S1-14; Supplemental Tables S1-10; Supplemental Methods; Supplemental Linkage File
REFERENCES


FIGURES WITH LEGENDS:

**Figure 1. Hyperglycemia induces robust metabolite alterations in tumor xenografts.**

(A) Unsupervised principal component analysis using the metabolite data obtained from xenografts grown in diabetic (D) and non-diabetic mice (ND). The plot shows data points for each of the MDA-MB-231, MDA-MB-468 and Hs578T xenografts and highlights the separation by diabetes status. (B) Heatmaps emphasizing the difference in intratumor metabolite abundance between diabetic and non-diabetic xenografts (FDR cutoff < 0.3 for inclusion of differential metabolites). The plots show the data from MDA-MB-231, MDA-MB-468 and Hs578T xenografts. (C) Venn diagram with 71 metabolites whose levels are altered by diabetes across MDA-MB-231, MDA-MB-468 and Hs578T xenografts (FDR < 0.05). Fifty-three of them were consistently up-regulated, 14 down-regulated in all xenografts of diabetic mice. (D) Intratumor levels of the diabetes markers, glucose and 1,5 anhydroglucitol (1,5 AG), in MDA-MB-231, MDA-MB-468 and Hs578T xenografts by diabetes status. Data represent mean ± SD of log transformed relative abundance levels (n=4 each group), with t-test for significance testing.
Figure 2. A distinct transcriptome profile in breast tumors of patients with diabetes. (A) Heatmap highlighting the difference in gene expression for breast tumors from diabetic (yes) and non-diabetic (no) patients (FDR < 0.05 for inclusion of differentially expressed transcripts, covariate-adjusted). (B) Enrichment of differentially expressed genes (diabetic vs non-diabetic, covariate-adjusted) in GSEA Hallmark gene sets (FDR < 0.25). Y-axis represents the enriched gene sets (either positive or negative), and X-axis represents the normalized enrichment scores (NES) for each gene set. (C) Enrichment of differentially expressed genes (diabetic vs non-diabetic, covariate-adjusted) in GSEA KEGG gene sets (FDR < 0.25).
Figure 3. Activity scores for key pathways altered in breast tumors of patients with diabetes. (A-F) Myogenesis and hedgehog signaling scores in either all, ER-positive or ER-negative tumors by diabetes status. (G) Hallmark DNA repair, (H) KEGG Base Excision Repair (BER), (I) KEGG Homologous Recombination, (J) KEGG Mismatch Repair, and (K) KEGG Nucleotide Excision Repair (NER) pathway scores in breast tumors by diabetes status. (A-K) Single-sample pathway scores were obtained from ssGSEA with adjustments for covariates (age, BMI, race, stage, and ER status). The significance of the diabetes status in influencing the activity scores was assessed via multivariable linear regression to control for covariates.
Figure 4. Diabetes and hyperglycemia promote mesenchymal and stem cell differentiation. (A-C) Tumor proliferation index in ER-positive and ER-negative breast tumors by diabetes status. Significance testing with Wilcoxon rank sum test. (D-F) Hallmark-annotated EMT pathway scores (ssGSEA-based and covariate-adjusted) in ER-positive and ER-negative tumors by diabetes status; Wilcoxon test. (G) Enrichment of differentially expressed genes (diabetic vs non-diabetic; covariate-adjusted) in GSEA gene set “HOLLERN_EMT_BREAST_TUMOR_UP”. Signature is up with diabetes. (H) Enrichment of differentially expressed genes (diabetic vs non-diabetic; covariate-adjusted) in GSEA gene set “LIM_MAMMARY_STEM_CELL_UP”. Signature is up with diabetes.
Figure 5. Hyperglycemia induces breast cancer cell migration, invasion and stemness. (A-C) Migration of breast cancer cells (MDA-MB-231, Hs578T, MDA-MB-468) under hyperglycemia. Shown are data for the 24-hour timepoint. Data represent mean ± SD of 4 replicates; t-test. (D-E) Hs578T and MDA-MB231 cells cultured under hyperglycemia develop an elongated morphology. Picture taken with ×200 magnification setting. (F-G) Quantitative analysis of the elongated cell morphology in Hs578T and MDA-MB231 cells cultured under hyperglycemia using the ImageJ software. Data represents average length of 100 cells from 5 different representative areas in each group; Wilcoxon test. (H) Matrigel invasion by Hs578T breast cancer cells under hyperglycemia. Shown are data for the 24-hour timepoint. Data represent mean ± SD of 5 replicates; t-test. (I) Matrigel invasion by MDA-MB-231 breast cancer cells under hyperglycemia. Shown are data for the 24-hour timepoint. Data represent mean ± SD of 5 replicates; t-test. (J) MDA-MB-231-LM2 cells harboring a stemness reporter were cultured +/- hyperglycemia. Number of SORE6+ cells among cultured MDA-MB-231-LM2-SORE6-mcherry breast cancer cells exposed to either 5 mM glucose (control) or hyperglycemia (25 mM glucose) for 48 hrs. Hyperglycemia increases the number of SORE6+ cells, which is indicative of increased stemness. Addition of the positive control compound, TRULI, a Lats1/2 kinase inhibitor, increases the stemness signal. We did not observe SORE6+ cells among the control vector cells (MDA-MB-231-LM2 mCMV-mcherry) when cultured +/- 25 mM glucose. Data represents mean ± SD. t test for statistical analysis.
Figure 6. Hyperglycemia induces oxidative stress in breast cancer cells. (A-D) FACS analysis shows increased mitochondrial superoxide production (with MitoSOX) in breast cancer cells cultured under hyperglycemia. Shown are representative flow cytometry experiments for the (A) Hs578T and (C) MDA-MB-231 cell lines. There is a shift towards increased MitoSOX under hyperglycemia. Quantification of FACS analysis data for Hs578T and MDA-MB-231 cells are shown in figure (B) and (D) respectively. MitoSOX fluorescence comparing control vs. hyperglycemia with 5 repeats; t-test. For normalization and display, we set control values as 1. (E-H) Superoxide radical scavenger, Mitotempo (200 µM), inhibits hyperglycemia-induced migration of Hs578T and MDA-MB-231 cells. Each time point shows mean ± SD of 4 replicates. ANOVA with post hoc test for statistical analysis. * P < 0.05, ** P < 0.01, *** P < 0.001
**Figure 7. Hyperglycemia increases DNA damage in breast cancer cells.** (A) Representative immunofluorescence images of γH2AX staining in Hs578T cells under hyperglycemia. Scale bar is 20 µm for γH2AX, DAPI, and merged images. (B) Quantification of γH2AX in Hs578T cells comparing control vs. hyperglycemia using ImageJ software. Data show mean ± SD of normalized fluorescence from 50 nuclei taken from five different areas for each group; t-test for significance testing. (C) Representative immunofluorescence images of 53BP1 staining in Hs578T cells under hyperglycemia. Shown scale bar is 20 µm. (D) Quantification of 53BP1 in Hs578T cells comparing control vs. hyperglycemia using ImageJ software. Data represents mean ± SD of the average percentage of localized 53BP1 expression in positive nuclei in each group, using n=5 images from each group and t-test.
Figure 8. Diabetes affects DNA repair capacity as evident by IPA analysis. (A-B) IPA analysis with 461 genes whose expression is commonly altered by diabetes/hyperglycemia in both patient tumors and xenografts. (A) Summary graph of the IPA analysis indicates activation of DNA damage signaling like “Formation of gamma H2AX nuclear focus” in presence of diabetes. Blue color indicates “inhibition” and red color indicate “activation” of a process. (B) Pathway enrichment analyses in IPA. Blue color indicates “inhibition” and red color indicate “activation” of a pathway/process by diabetes. “Role of BRCA1 in DNA damage response” is the top pathway indicated to be inhibited by diabetes.
Figure 9. Hyperglycemia impairs DNA repair capacity in breast cancer cells. (A-D) Decreased NHEJ DNA repair capacity under hyperglycemia. Breast cancer cells (Hs578T and MDA-MB-231) cultured under high glucose showed a decrease in the non-homologous end joining (NHEJ) DNA repair capacity as measured by a reporter assay (see methods). A decrease in GFP-positive cells in the high glucose groups corresponds to a decrease in DNA repair capacity. Figure A and C represent the FACS analysis of Hs578T and MDA-MB-231 cells, respectively. The graphs show the quantification of FACS analysis-based data for Hs578T and MDA-MB-231 cells in Figure B and D respectively. Data represent mean ± SD of the % GFP positive cells comparing hyperglycemia (25 mM glucose) vs. control (5 mM glucose, n = 4 each), with significance testing by t-test.
Figure 10. Hyperglycemia increases sensitivity to drugs targeting the DNA damage repair pathway.

Increased sensitivity of 5 human breast cancer cell lines to DNA damage repair inhibitors under hyperglycemia. Shown are the IC50 values as mM concentrations for AZD7762, berzosertib, and etoposide comparing cells cultured under control conditions (5 mM glucose) versus high glucose (25 mM glucose = hyperglycemia). Panel at the right shows the sensitivity to 1, 10 and 20 µM concentrations of olaparib comparing control versus high glucose with normalized BrdU incorporation (absorbance at 370 nm) as readout. Cell viability was measured with the BrdU incorporation assay. Data are shown as mean ± SD with Wilcoxon rank sum test for significance testing.
Figure 11. Mutational signatures in breast cancer patients with diabetes. (A) Mutational trinucleotide frequency distribution in breast tumors from patients without diabetes (ND, top), with diabetes developing after the tumor resection (pre-D, center), and with diabetes at the time of tumor resection (D, bottom). Due to strand complementarity, two equivalent sets of annotations are possible, either based on the substitution of purines (blue) or pyrimidines (red). There are no obvious differences by diabetes status. (B) Heatmap showing signature age-adjusted weights by diabetes status (top bar) obtained from non-negative least squares mapping of individual samples (columns) vs reference signatures (rows) from the COSMIC catalogs and the Compendium of Mutational Signatures of Environmental Agents. Yellow color indicates upregulation of a signature in a sample. Diabetic (D), n = 38; diabetic after surgery (pre-D), n = 7; non-diabetic (ND), n = 71.
Figure 12. Mutational landscape of breast cancer patients with diabetes. Oncoplot showing 17 mutated genes (rows) across 116 subjects (columns) split by diabetes status. Within each group, subjects were ordered in waterfall fashion. Included genes are those mutated in > 5% of the samples. Diabetic (D), n = 38; diabetic after surgery (pre-D), n = 7; non-diabetic (ND), n = 71.