Microenvironment and Immunology

Imaging the Unfolded Protein Response in Primary Tumors Reveals Microenvironments with Metabolic Variations that Predict Tumor Growth

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Abstract

Cancer cells exist in harsh microenvironments that are governed by various factors, including hypoxia and nutrient deprivation. These microenvironmental stressors activate signaling pathways that affect cancer cell survival. While others have previously measured microenvironmental stressors in tumors, it remains difficult to detect the real-time activation of these downstream signaling pathways in primary tumors. In this study, we developed transgenic mice expressing an X-box binding protein 1 (XBP1)-luciferase construct that served as a reporter for endoplasmic reticulum (ER) stress and as a downstream response for the tumor microenvironment. Primary mammary tumors arising in these mice exhibited luciferase activity in vivo. Multiple tumors arising in the same mouse had distinct XBP1-luciferase signatures, reflecting either higher or lower levels of ER stress. Furthermore, variations in ER stress reflected metabolic and hypoxic differences between tumors. Finally, XBP1-luciferase activity correlated with tumor growth rates. Visualizing distinct signaling pathways in primary tumors reveals unique tumor microenvironments with distinct metabolic signatures that can predict for tumor growth.

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Introduction

The tumor microenvironment remains a poorly characterized stress on cancer cells, reflecting several variables including stromal cell interactions, hypoxia, and nutrient deprivation (1–4). These microenvironmental pressures interact during tumorigenesis to promote or inhibit tumor growth. Stromal cells may receive or transmit signals from or to cancer cells to promote angiogenesis, invasion, and metastasis (5). Hypoxia activates progrowth signaling pathways and destabilizes the cancer cell genome to enhance tumorigenesis (6, 7). By contrast, nutrients may become limited in the tumor microenvironment, potentially inhibiting cell growth and/or activating cell death pathways (7). Many groups have developed ways to quantitate these stresses in the tumor microenvironment. However, it remains unclear how much these microenvironmental stressors activate downstream signaling pathways in cancer cells. By understanding how the tumor microenvironment may affect signaling pathways among similar primary tumors, we can gain insights into cancer biology and individualizing cancer therapy.

One putative marker for the tumor microenvironment is endoplasmic reticulum (ER) stress, which can be triggered within cancer cells by hypoxia or nutrient deprivation (8, 9). These stressors lead to the abnormal accumulation of proteins within the ER. Cells respond by activating the unfolded protein response (UPR) that regulates the transcription and translation of genes to maintain ER homeostasis (10). The UPR encompasses a three-pronged signaling network that includes the inositol-requiring 1α (Ire1α)-X-box binding protein 1 (XBP1) pathway. The UPR interacts to promote cell survival or, if the ER stress is too great or too long, to activate cell death. Several groups have shown that the UPR is activated in tumors including high-grade breast, colorectal, and pancreatic carcinomas (11–14). In addition, UPR proteins can promote tumor growth in both transplantable and primary tumor models (15–18). Thus, the UPR may link the tumor microenvironment to cancer cell death and survival, as well as serve as a marker for the microenvironmental stressors occurring in primary tumors.

Here, we monitored the microenvironment in primary tumors by developing a transgenic mouse model to report XBP1-luciferase (XBP1-luc) activity as a marker for ER stress. XBP1-luc activity was heterogeneous among similarly sized primary tumors within the same mouse, indicating that primary tumors had unique microenvironments with variable levels of ER stress. This unique XBP1-luc activity inversely correlated with glucose avidity and directly correlated with hypoxia and tumor growth. Together, these
distinct microenvironments may explain differences in growth and metabolism in similar primary tumors.

**Materials and Methods**

**Tumor cell lines and reagents.** HT1080 cells were obtained from the American Type Culture Collection. For generation of tumor cell cultures, cells were isolated from mice and directly placed into culture. Cells were maintained at 37°C with 5% CO₂ in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics. Tunicamycin, 2-deoxy-D-glucose (2-DG), and propidium iodide were obtained from Sigma. Rabbit anti-IkB, rabbit anti-phospho eIF2α, and mouse anti-CHOP antibodies were obtained from Cell Signaling. Rabbit anti-XBP1 was obtained from Abcam. Goat anti-luciferase antibodies were obtained from Promega, and mouse anti-luciferase antibodies were obtained from AdD Serotec. Goat anti–carbonic anhydrase IX (CA-IX) antibodies were obtained from R&D systems.

**XBP1L-luc and cytomegalovirus-luc vectors and cell lines.** HT1080 cells expressing the XBP1-luc (8), ATF4-luc (19), and cytomegalovirus (CMV)-luc (19) constructs have been previously described. For hypoxia treatments, cells were incubated for in <0.1% O₂ concentrations (Sheldon Corp.).

**Luciferase assays.** Cells were seeded at 50% to 70% confluence and treated accordingly. For ex vivo analysis of luciferase activity, tumors were isolated and diced into small fragments. Cells were lysed in Bright Glo lysis buffer (Promega) for 20 min and luciferase activity was assessed using a Berthold LB 96 luminometer or Monolight 2010 (Analytic Luminescence Laboratory).

**Mice.** FVB/N-Tg(MMTV-PyVT)634 Mul/J (Tag), FVB/N-Tg (MMTVneu202Mul/J (Her2), FVB/N, and severe combined immunodeficient (SCID) mice were purchased from The Jackson Laboratory. Mice were housed at Stanford University Research Animal Facility and maintained according to Stanford Institutional Care and Use Committee guidelines.

**Generation of XBP1-luc transgenic mice.** The XBP1-luc transgene was injected into FVB/N oocytes by Stanford Transgenic Animal Core Facility and a single founder line was maintained.

**Bioluminescent imaging.** Luciferase activity was measured noninvasively using the IVIS imaging system (Caliper LifeSciences). Mice were injected i.p. with luciferin (300 mg/kg dissolved in PBS; Caliper) and anesthetized via inhaled 3% isoflurane (Abbott Laboratories Ltd.). Exposure time ranged from 10 s to 10 min. All images were analyzed using Living Image software (Caliper) with a binning of 10. *In vivo* bioluminescent signal was quantified by taking the average bioluminescent signal, which is photon count per second per square centimeter. For comparisons between mice, the bioluminescent signal of the tumors was normalized to adjacent skin.

RGD and 2-DG avidity was determined using spectral fluorescence imaging via the Maestro In-Vivo imaging system (CRi). IRDye 800CW 2-DG, RGD, and the unlabeled dye were obtained from Li-Cor and injected i.v. into tumor-bearing mice. After 24 to 48 h, mice were then imaged using near-IR excitation/emission filter sets and images were obtained from 780- to 900-nm wavelengths at 10-nm intervals. Exposure time ranged from 250 to 1,500 ms.

**Histology.** Tumors were isolated and immediately embedded in optimum cutting temperature compound (Sakura Finetek). Cryosections were generated and blocked with a 5% mixture and goat and donkey anti-serum. Sections were then stained with the respective antibodies according to the manufacturer’s instructions. Antibody binding was detected with AF555-labeled goat anti-mouse, AF555-labeled goat anti-rabbit, AF488-labeled donkey anti-goat, or AF488-labeled goat anti-rabbit.

**O₂ measurements.** Tumor oxygen measurements were assessed using Oxylite pO₂ E-series probe (Oxford Optronix). Briefly, tumor-bearing mice were anesthetized and Oxylite probe was inserted into the tumor and allowed 30 to 60 s to equilibrate. Average tumor oxygenation was assessed from the average of three separate measurements for each tumor.

**In vivo tumor transplantation and tumor growth.** Solid tumor fragments were harvested from Tag-luc primary tumors and diced into small tumor fragments. The fragments were injected using a 10-gauge trochar. For injection of SCID mice, 5 × 10⁶ cultured Tag-luc cells were injected. Tumor volume was calculated as follows: tumor volume (mm³) = (a x b x c) / 2.

**Statistics.** Tumor incidence curves were estimated with the Kaplan-Meier method using Cox log-rank method for significance. Two-sided independent Student’s t tests were done to analyze the results of *in vitro* luciferase assays, *in vivo* bioluminescence, or tumor growth time points. *In vivo* bioluminescence, *in vitro* luminescence, tumor growth, and 2-DG uptake were analyzed with ANOVA.

**Results**

**Primary tumors that developed in transgenic mice had increased XBP1-luc activity.** During the UPR, activation of XBP1 occurs through a novel splicing mechanism where Ire1α removes 26 nucleotides from the XBP1 mRNA (20–23). To study ER stress in tumors, we generated an XBP1-luc reporter construct (Fig. 1A, top). We fused the cDNA for the first 208 amino acids of the human XBP1 to firefly luciferase (XBP1-luc). This XBP1 sequence has been used in similar XBP1 reporter models and contained the hydrophobic domain-2 that regulates XBP1 activation (24–33). Under non–ER-stress conditions, the XBP1-luc mRNA remains unspliced, with an in-frame stop codon preventing expression of the downstream luciferase gene. Under ER stress conditions, the XBP1-luc transcript is spliced, shifting the stop codon out of frame and allowing expression of the luciferase gene. We stably expressed this construct in HT1080 cells (Fig. 1A, bottom). Similar to the UPR reporter ATF4-luc, XBP1-luc cells strongly induced luciferase activity under conditions of ER stress such as tunicamycin treatment, glucose depletion, or hypoxia. XBP1-luc activity required Ire1α, consistent with its requirement for splicing to report ER stress (Supplementary Fig. S1).
We generated XBP1-luc transgenic mice and bred them to breast cancer–prone Tag mice to generate the double transgenic Tag-luc mouse (Fig. 1B and C) Supplementary Fig. S2A). Tag mice express the middle T antigen under the control of the mouse mammary tumor virus (MMTV) promoter and develop multiple primary mammary carcinomas at a mean interval of 42 days (34). Normal skin fibroblasts isolated from Tag-luc mice constitutively expressed the XBP1-luc fusion mRNA transcript (Supplementary Fig. S2B). Under hypoxia, the XBP1-luc transcript was spliced similarly to the endogenous XBP1 transcript and elicited luciferase activity (Supplementary Fig. S2C and D).

Tag-luc double transgenic mice developed primary tumors at similar rates as the control Tag mice (Fig. 1B; for females, P = 0.72; for males, P = 0.07). Imaging of tumor-bearing Tag-luc mice revealed that regions corresponding to the mammary tumors possessed bioluminescent signal (Fig. 1C). By contrast, primary tumors arising in control Tag mice possessed background signal. The single transgenic XBP1-luc mouse (Fig. 1C) had basal bioluminescent signal in the spleen, which was confirmed with dissection (Supplementary Fig. S3) and consistent with previous reports on the activity in that organ using a nearly identical XBP1-GFP transgenic reporter mouse (27). Similar to the XBP1-GFP transgenic reporter mouse, basal bioluminescent activity in our XBP1-luc mouse was also detectable in the pancreas and liver (data not shown). Non–tumor-bearing Tag-luc mice did not have appreciable bioluminescent signal along the mammary chains. In tumors from Tag-luc mice, luciferase colocalized with XBP1, as well as other UPR proteins including BiP, phospho-eIF2α, and CHOP, whereas in control Tag tumors, luciferase was not detected (Fig. 1D). Therefore, primary tumors possessed XBP1-luc activity that correlated to regions of ER stress in primary tumors.

Individual primary tumors had unique levels of XBP1-luc activity that were modulated by the tumor microenvironment. Overall, Tag-luc tumors possessed bioluminescent signal that was 9.36 ± 0.08-fold greater than that in the background skin and significantly greater than that in control tumors (Fig. 2A, left). Tumors isolated ex vivo by dissection from Tag-luc transgenic mice had 14.5-fold greater luciferase activity than tumors isolated from control Tag transgenic mice (Fig. 2A, middle). Furthermore, in vivo bioluminescence signal significantly correlated with the in vitro luciferase activity of each individual tumor (Fig. 2A, right). Overall, primary breast tumors possessed XBP1-luc activity that was significantly increased above background. However, we observed that each tumor within the same mouse possessed a distinct bioluminescent signal (Fig. 2B). The heterogeneity of XBP1-luc activity in different primary tumors growing in the same mouse remained a consistent phenomenon in vivo and ex vivo (Fig. 2C; Supplementary Fig. S4). By contrast to Tag-luc mice, tumors arising in Tag mice (Fig. 2B, left) did not possess greater bioluminescence signal than nonneoplastic tissues.

It remained unclear whether the heterogeneous XBP1-luc activity in each primary tumor reflected the variability of the tumor microenvironment or was a hereditary feature of the tumor. We defined tumors with bioluminescence signal above background skin as “Hi tumors” and those with bioluminescence signal similar to background skin as “Lo tumors.” In vivo differences in XBP1-luc activity were not maintained in cell culture, as both Hi and Lo cultured tumor cells showed background bioluminescent activity (Fig. 3A). After tunicamycin treatment to induce ER stress, XBP1-luc activity in Hi and Lo cultured tumor cells was induced to similar levels, indicating that both tumor phenotypes retained similar responsiveness to ER stress.

To confirm that primary tumors developed variable and nonheritable levels of ER stress, we harvested primary tumors from mice bearing both Hi and Lo tumors and transplanted them s.c. into the flanks of syngeneic mice (Fig. 3B). The Hi primary tumor (indicated in red) was transplanted into the upper left flank, whereas the Lo primary tumors (indicated in yellow) were transplanted in the remaining three quadrants of a single mouse. Fourteen days after transplantation, we observed that both Hi and Lo tumors had similar levels of XBP1-luc bioluminescent signal (Fig. 3B and C). Furthermore, the differences in luciferase signal between Hi and Lo tumors were not due to differences in reporter mRNA expression (Supplementary Fig. S5A). Finally, Hi tumors had increased endogenous XBP1 splicing, paralleling the splicing of the reporter XBP1-luc, as well as increased expression of BiP, confirming increased ER stress in tumors with higher bioluminescent signal (Fig. 3D; Supplementary Fig. S5B). Therefore, ER stress caused by the tumor microenvironment was unique to each primary tumor and reflected the variation of microenvironmental stress.

Increased XBP1-luc activity was regulated by the loss of glucose utilization in vivo. To elucidate what factors may mediate these differences of XBP1-luc activity, we injected tumor-bearing Tag-luc mice with labeled RGD peptide or 2-DG probes to measure angiogenesis or glucose uptake, respectively. Compared with mice injected with the RGD probe or the dye alone, primary tumors with higher XBP1-luc activity (red arrows) possessed significantly lower 2-DG uptake [Fig. 4A (top) and B]. Conversely, tumors with lower XBP1-luc activity (yellow arrows) had higher 2-DG uptake. By contrast, the level of XBP1-luc activity did not significantly correlate with RGD uptake or microvessel density in tumors (Supplementary Fig. S6A and B).

To confirm that differences in 2-DG uptake were not due to genetic differences or tumor size, cells from a single Tag-luc tumor were injected at multiple sites in a single mouse. Tumors grew to similar sizes and showed heterogeneous XBP1-luc signal (Fig. 4A, bottom). As with primary tumors, transplanted tumors with higher XBP1-luc activity (red arrows) had lower 2-DG uptake. Tumors with lower XBP1-luc activity (yellow arrows) had higher 2-DG uptake. Thus, XBP1-luc activity in the microenvironment inversely correlated with glucose uptake in tumors.

Because the XBP1-luc activity in tumors inversely correlated with glucose uptake, we determined whether inhibition of glucose utilization could modulate XBP1-luc activity in primary tumors. Indeed, tumor cells cultured in glucose-depleted medium had 27.2-fold more luciferase activity than control treated cells (Fig. 4C). We treated mice with 2-DG to mimic a state of glucose deprivation. Tag-luc
Figure 1. Primary breast carcinomas had increased XBP1-luc activity. A, top, schematic of the XBP1-luc reporter vector. Bottom, the XBP1-luc reporter vector induced luciferase activity in response to ER stress. HT1080 cells expressing the XBP1-luc construct or an ATF4- or a CMV-driven luciferase were subjected to 10 μg/mL tunicamycin (Tun), glucose depletion, or severe hypoxia for 48 h to induce ER stress. B, incidence of breast carcinomas in Tag mice was similar to that in Tag-luc double transgenic mice. C, in Tag-luc transgenic mice, primary breast tumors had increased bioluminescence. Top, photograph of breast tumors; arrows, tumors along the mammary chain. Middle, photograph of Luc, FVB, tumor-bearing Tag-luc, tumor-bearing Tag, and non–tumor-bearing Tag-luc mice. Red outline, tumor areas. Bottom, overlay of bioluminescence. Note that the bioluminescence activity was detected only in tumor-bearing Tag-luc mice. D, luciferase localized to XBP1 and other ER stress proteins in Tag-luc mice tumors. Frozen sections were stained with anti-luciferase and anti-XBP1, anti-BIP, anti–phospho-eIF2α, or anti-CHOP.
tumors treated with 2-DG had a 1.78-fold increase in bioluminescence compared with control mice (Fig. 4D). Therefore, the heterogeneity of ER stress in primary tumors correlated with the loss of glucose uptake because inhibition of glucose utilization increased ER stress.

**Hypoxia modulated XBP1-luc signal in spontaneous tumors.** To determine if XBP1-luc activity was regulated by hypoxia, we analyzed Tag-luc tumors for XBP1-luc protein in hypoxic areas of the tumor. Indeed, luciferase activity colocalized with the hypoxic marker CA-9 and with lower $pO_2$ levels, indicating that XBP1 splicing occurred in hypoxic areas (Fig. 5A). To confirm that XBP1 splicing could occur in hypoxic microenvironments, we isolated primary Tag-luc breast tumors and cultured the cells for 5 to 10 days. After 48 hours of hypoxia, Tag-luc tumor cells had 58.0-fold more luciferase activity than normoxic cells (Fig. 5B), indicating that Tag-luc tumors spliced XBP1 under hypoxic conditions.

To confirm that hypoxia regulated XBP1-luc activity, we treated mice with repeated injections of dichloroacetic acid (DCA), which increases tumor oxygen consumption to mimic...
a state of increasing hypoxia (35). Primary Tag-luc tumors increased bioluminescent signal by 2.0-fold after repeated DCA injections, confirming that hypoxia can regulate XBP1-luc activity (Fig. 5C). DCA alone did not directly induce ER stress because HT1080 cells expressing XBP1-luc or ATF4-luc did not induce luciferase activity in response to DCA (Fig. 5D). Therefore, primary tumors in Tag-luc mice could induce ER stress in response to hypoxic microenvironments.

Higher XBP1-luc activity correlated with faster doubling times. Various UPR proteins are important to the growth of primary tumors (15, 17, 18). Because XBP1-luc activity was heterogeneous among similar primary tumors, we determined if differences in XBP1-luc activity served as a marker for differences in tumor growth rates. Indeed, tumors with higher XBP1-luc signal had significantly faster doubling times compared with tumors with lower XBP1-luc signal (Fig. 6A). We then serially imaged Tag-luc mice over time to detect changes in XBP1-luc signal. We observed that changes in XBP1-luc signal increased, remained stable, or decreased (Fig. 6D). Compared with tumors with stable or increasing signal, tumors with decreasing signal also had slower doubling times (Fig. 6C). By contrast, XBP1-luc activity did not correlate with tumor size, indicating that XBP1-luc activity was not simply a reflection of tumor mass (Fig. 6D). Even when tumors with XBP1-luc signal that was more than 2-, 5-, or 15-fold above background were only included in analysis, the lack of correlation between bioluminescent signal and tumor size remained (data not shown).

Figure 3. Tumors with low XBP1-luc activity develop increased signal after transplantation. A, high in vivo XBP1-luc activity was not maintained in vitro. Primary tumors with Hi or Lo XBP1-luc activity were cultured in vitro, causing luciferase activity to return to background levels. Tunicamycin induced XBP1-luc activity to similar levels in cultured Hi and Lo tumors. B, primary tumors with low XBP1-luc activity increased bioluminescence after transplantation. Left, Tag-luc mice bearing synchronous Hi (red arrows) and Lo (yellow arrows) tumors. Right, primary tumors with Hi activity were transplanted into the left upper flank (red arrows) and primary tumors with Lo activity were transplanted into the remaining quadrants (yellow arrows). Bioluminescence was assessed 14 d after transplantation. C, quantitation of primary and transplanted tumors in B. Similar results were seen in four independent experiments using 20 mice with 71 transplanted tumors. D, left, mRNA from Hi and Lo tumors was isolated and subjected to reverse transcription-PCR analysis for the spliced endogenous XBP1, XBP1-luc, and BiP. Right, comparison of the ratio of spliced/unspliced isoforms of endogenous XBP1 and XBP1-luc.
To confirm that primary tumors possessed XBP1-luc activity, we also bred the breast cancer–prone Her2 [FVB/N-Tg(MMTVneu202Mul/J); ref. 36] mice to the XBP1-luc reporter mice (Her2-luc). As with Tag-luc cells, cells from Her2-luc tumors spliced the XBP1-luc reporter similar to the endogenous XBP1 mRNA and induced luciferase activity under ER stress (Supplementary Fig. S2B–D). Her2-luc mice developed tumors at similar rates as the Her2 controls (Supplementary Figure 4).

Loss of glucose uptake and utilization resulted in increased XBP1-luc activity in primary and transplanted tumors. A, XBP1-luc activity in primary tumors correlated with low glucose avidity. Tag-luc mice bearing primary tumors (top) or SCID mice bearing transplants of the same tumor (bottom) were injected with fluorescent 2-DG or control dye; 24 h later, mice were imaged for XBP1-luc activity and 2-DG or control dye uptake. Red arrows, Hi tumors; yellow arrows, Lo tumors. B, for mice imaged in A, the XBP1-luc signal was plotted against the corresponding 2-DG signal. C, XBP-luc activity in primary tumor cells increased during glucose deprivation in vitro. D, top, chemical inhibition of glucose utilization in vivo increased ER stress in tumors. Tag-luc mice were treated with vehicle or 2 mg of 2-DG i.p. and bioluminescence was assessed after 12 h. Arrows, palpable tumors. Bottom, quantitation of bioluminescence. Columns, mean; bars, SEM.
Fig. S7A; Supplementary Table S1). Tumors arising in Her2-luc mice possessed bioluminescence signal that colocalized to areas of ER stress as indicated by XBP1 and BiP (Supplementary Fig. S7B and C). However, compared with Tag-luc mice, XBP1 signal in Her2 tumors was less frequent and intense (Supplementary Fig. S7D). Ex vivo luciferase activity of Her2-luc tumors was statistically greater than that of control tumors (Supplementary Fig. S7E). Therefore, in vivo and in vitro XBP1-luc activities were detected in two different primary breast tumor models.

Discussion

We observed that primary tumors possessed unique levels of ER stress that were regulated by distinct microenvironmental factors. Furthermore, these differences in ER stress reflected the metabolic profile of tumors as determined by glucose uptake and hypoxia. Finally, XBP1-luc activity also predicted for faster tumor growth. In our model, tumorigenesis was driven by the expression of the middle T antigen in mammary epithelium, which may not reflect the natural...
history of human breast cancers. Nevertheless, XBP1-luc activity was detected in a second Her2/neu mammary tumor model possessing a more physiologic oncogenic event and was consistent with previous findings (11–13). Although our reporter likely does not detect extremely low levels of XBP1 splicing that may occur in Lo tumors and normal tissues, we did observe XBP1-luc signal under conditions of ER stress sufficient for endogenous XBP1 splicing. Thus, the limitation in the sensitivity of this model did not preclude the relative comparison of ER stress between tumors. Because we examined tumors of the same histologic subtype driven by the same oncogenic event, we expect that any differences in XBP1-luc signal were likely due to the biological properties of the tumors and not an artifact of our model.

We observed that XBP1 splicing reflected ER stress imposed by the tumor microenvironment based on several findings. First, tumors with increased XBP1-luc bioluminescence signal in vivo possessed only baseline in vitro luciferase activity under nonstressed conditions. Second, XBP1-luc activity was gained or lost on in vivo passage of primary tumors and, therefore, reflected the situational environment to which the cancer cells were subjected. Finally, treatment of mice with agents known to modulate the microenvironmental stresses of hypoxia or glucose deprivation also regulated XBP1-luc activity. Previous investigators have manipulated UPR pathways to show an effect on tumor growth in vivo but not in vitro, suggesting an interplay between ER stress and the tumor microenvironment (15, 18). However, it remained unclear whether the activation of the UPR in primary tumors was a hereditary change acquired by tumor cells or a response to microenvironmental stress. By studying XBP1-luc activity under different tumor conditions, our data showed that XBP1 is a critical marker of ER stress occurring in the tumor microenvironment.

Using XBP1 splicing as a surrogate for ER stress in the tumor microenvironment, we observed that primary tumors displayed different levels of XBP1-luc activity that were not maintained after in vivo or in vitro passage. It is likely that certain aspects of the tumor microenvironment remain similar between the primary and the transplanted tumor settings (3–5, 37). In contrast, our data showed that other aspects of the tumor microenvironment are unique to each primary tumor and were not maintained once tumors were introduced into a new environment. It has previously been shown that similar tumors possessed distinct metabolic (38–40), hypoxic (41), and interstitial pressure (42) profiles in their tumor microenvironments. Whereas the exact mechanisms that regulate these differences remain to be elucidated, our data suggested that ER stress caused by the tumor microenvironment reflected glucose avidity and hypoxia within tumors. Previous reports may not have observed this because the majority of these systems dealt with a single primary tumor in a single mouse. Thus, our unique model in which several tumors arise in a single mouse allowed us to show that stress...
pathways important for tumor growth may be distinct among very similar tumors.

The correlation of low glucose avidity in tumors with increased ER stress paralleled recent clinical observations in human tumors. Our results were consistent with the in vitro and in vivo observations that glucose deprivation is a potent inducer of ER stress. Moreover, other tumor microenvironmental stresses such as hypoxia, in combination with low glucose, would further exacerbate underlying ER stress within tumors. Our results suggested that specific biological pathways may be detected and targeted by measuring glucose uptake in tumors, which is done clinically through positron emission tomography (PET) imaging. Because PET imaging has correlated with treatment response (43, 44), it is interesting to speculate how ER stress and the associated differences in glucose uptake correlate with tumor biology and response to therapy.

In addition to glucose deprivation, we also observed that prolonged hypoxia induced XBP1-luc activity in vitro and correlated with bioluminescent signal in vivo. However, the relative contributions of hypoxia and glucose avidity to ER stress remained difficult to dissect. Furthermore, increased XBP1-luc activity within tumors is a global indication of ER stress reflecting the contribution of not only hypoxia but also glucose and nutrient deprivation. Finally, because luciferase activity is dependent on the presence of O₂, increasingly low O₂ content in tumors may have underestimated true XBP1-luc activity and reduced the significance of this in vivo correlation. Therefore, it is difficult to determine the relative contribution of a single stressor to XBP1-luc signal.

We observed that the doubling time of tumors directly correlated with XBP1-luc activity. First, within the same mammary carcinoma model, tumors with higher XBP1-luc activity had faster doubling times, whereas tumors with lower XBP1-luc activity had slower doubling times. Because it is difficult to genetically manipulate the growth rates of primary tumors in vivo, we can only correlate XBP1-luc activity to tumor growth in Tag-luc mice. Nevertheless, in a second mammary tumor model, Her2, tumors that contained inherently slower doubling times possessed lower XBP1-luc activity compared with Tag-luc mice (Supplementary Table S1; Supplementary Fig. S7D and E). Whereas the relationship between lower luciferase signal and tumor growth in the Her2 model compared with the Tag model may be an overinterpretation of the data, these observations are consistent with other reports that tumors with intact ER stress responses grow faster. Furthermore, our data suggest that faster-growing tumors likely have less time to adapt to their blood supply leading to increased hypoxia and nutrient deprivation, a microenvironment consistent with increased ER stress.

Although tumor growth correlated with higher XBP1-luc signal, our observations did not address whether increased XBP1 activity caused increased tumor growth, or increased tumor growth caused more ER stress. Our data more likely suggest the latter because increased tumor growth caused more ER stress associated with increased hypoxia and less glucose uptake as the tumor outgrows its blood and nutrient supply. Given these microenvironmental stresses, tumors are also more likely to have increased apoptotic proteins such as CHOP and increased cell death. Indeed, we observed that XBP1-luc colocalized to CHOP expression and tumors with higher XBP1-luc signal had increased cell death (data not shown). This parallels previous experimental and clinical observations that faster-growing tumors had more necrosis and high cell loss factors (45). It is important to note that XBP1 activation per se did not likely lead to cell death. Rather, such an environment may have activated a separate ATF4-CHOP pathway resulting in cell death. Nevertheless, intact UPR pathways are important for cell growth because loss of the ER stress protein BiP in spontaneous mammary carcinomas resulted in slow-growing tumors (17). In addition, cancer cells deficient in ATF4 or XBP1 grew slower than xenograft tumor models (15, 18). Finally, ER stress and UPR activation were associated with higher-grade breast carcinomas, consistent with the observation that faster-growing tumors are under more ER stress (46). Therefore, ER stress is likely a marker for faster-growing tumors experiencing increased microenvironmental stressors. Thus, increased XBP1 activation may be a consequence of faster tumor growth rate rather than an enabling event for increased tumor proliferation.

In conclusion, we have developed a novel transgenic model to study ER stress caused by the tumor microenvironment of spontaneous breast tumors. This aspect of the tumor microenvironment is distinct between primary tumors and reflects the metabolic properties and growth rates of tumors. Understanding the fundamental biological processes that contribute to tumor microenvironmental stresses will result in the development of cancer therapies that target the tumor microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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