Marginal zinc intake reduces the protective effect of lactation on mammary gland carcinogenesis in a DMBA-induced tumor model in mice

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Abstract. Breastfeeding can reduce breast cancer risk; however, unknown factors modify this protective effect. Zinc (Zn) modulates an array of cellular functions including oxidative stress, cell proliferation, motility and apoptosis. Marginal Zn intake is common in women and is associated with breast cancer. We reported that marginal Zn intake in mice leads to mammary gland hypoplasia and hallmarks of pre-neoplastic lesions. In the present study, we tested the hypothesis that marginal Zn intake confounds the protective effect of lactation on breast cancer. Nulliparous mice fed control (ZA, 30 mg Zn/kg) or a marginal Zn diet (ZD, 15 mg Zn/kg), were bred and offspring were weaned naturally. Post-involution, mice were gavaged with corn oil or 7,12-dimethylbenz(a)anthracene (DMBA, 1 mg/wk for 4 weeks) and tumor development was monitored. A ZD diet led to insufficient involution, increased fibrosis and oxidative stress. Following DMBA treatment, mice fed ZD had higher oxidative stress in mammary tissue that correlated with reduced levels of peroxiredoxin-1 and p53 and tended to have shorter tumor latency and greater incidence of non-palpable tumors. In summary, marginal Zn intake creates a toxic mammary gland microenvironment and abrogates the protective effect of lactation on carcinogenesis.

Introduction

Breast cancer is the most common cancer in women and the second leading cause of mortality among women in the USA, with ~40,000 deaths annually (American Cancer Society; http://www.cancer.org/research/cancerfactsstatistics/breast-cancer-facts-figures). One modifiable factor that is associated with reduced incidence of breast cancer is breastfeeding. However, while multiple meta-analyses show that breastfeeding reduces the risk for breast cancer and the protective effect is correlated with prolonged breastfeeding duration (1-3), protection is not always demonstrated (4). As a result it has been proposed that additional factors may modify the protective effect of breastfeeding on carcinogenesis.

One important factor is one's diet. We previously established that a marginal reduction in dietary zinc (Zn) during pregnancy and lactation in mice leads to insufficient mammary gland expansion (hypoplasia) and lactation insufficiency (5). More recently, we found that a marginal Zn reduction creates a toxic microenvironment in the mammary gland that includes hallmarks of pre-malignant lesions (6). Collectively, these studies suggest that marginal Zn intake may reduce protection from breast cancer. These observations in pre-clinical models have important implications for breast health in women, as 60-80% of women of reproductive age do not consume adequate Zn (7-9). In fact, several recent reports in women suggest a relationship between Zn deficiency and breast cancer (10,11). In addition, while serum Zn levels are inversely associated with malignancy (10), Zn accumulates in human breast tumors (12) and breast cancer cell lines (13), implicating Zn dysregulation in carcinogenesis, progression and metastasis.

Zn plays a structural, catalytic or regulatory role in ~10% of the proteome, regulating ~3,000 proteins and >300 different biological processes including proliferation, apoptosis, autophagy, motility, antioxidant defense and cell signaling (reviewed in ref. 14). Therefore, defects in Zn management have major implications for cell function and tissue health. For example, Zn accumulation in the breast increases oxidative stress (6), which can dyseregulate cell signaling and energy metabolism driving carcinogenesis (15). Secondly, Zn accumulation may underlie dysfunction in p53, a redox-active transcription factor that positively regulates expression of antioxidant genes to protect cells from damaging levels of oxidative stress, DNA damage and mutagenesis (16). In addition, many antioxidant systems are impaired by Zn depletion or

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exposure (17), thus, Zn accumulation may reduce antioxidant capacity. Finally, Zn accumulation may affect the activity of key enzymes. For example, matrix metalloproteinases (MMPs) are Zn-dependent proteins, thus, increased Zn accumulation may augment MMP activity and potentiate carcinogenesis (reviewed in ref. 18).

Cellular Zn management is coordinated by two different gene families of Zn transporters; ZIP proteins (*SLC39A*; ZIP1-14) that import Zn into the cytoplasm, and ZnT proteins (*SLC30A*; ZnT1-10) that export Zn from the cytoplasm. Two Zn transporters of particular interest in breast cancer are ZIP6 (19-22) and ZnT2 (13,23). ZIP6 imports Zn across the cell membrane into cells (24) and is upregulated in response to high glucose (25). Hogstrand *et al* (26) recently showed that overexpression of ZIP6 in MCF7 cells activates epithelial-to-mesenchymal transition through activation of Snail and loss of E-cadherin. However, increased ZIP6 protein abundance in breast tumors is associated with better prognosis (27), which is consistent with studies showing that ZIP6 attenuation in T47D cells activates epithelial-to-mesenchymal transition (20). On the other hand, ZnT2 imports Zn into vesicles for sequestration to protect against Zn toxicity and associated oxidative stress (28). ZnT2 is overexpressed in non-invasive T47D cells sequestering Zn in vesicles, and attenuation of ZnT2 results in oxidative stress and cell death (13). In contrast, ZnT2 is underexpressed in invasive MDA-MB-453 cells, and ZnT2 overexpression and Zn vesicularization restricts invasion (23). Collectively, this suggests that alterations in expression of key Zn transporters may underlie dysregulation of cellular Zn metabolism and may be important determinants of carcinogenesis, progression or metastasis.

In the present study, we report that the chronic consumption of a marginal Zn diet throughout an entire pregnancy-lactation cycle increased oxidative stress, inflammation and fibrosis in the mammary gland, and impaired post-lactational involution in mice. Moreover, following exposure to a chemical carcinogen, marginal Zn intake promoted Zn hyperaccumulation and oxidative stress, which was associated with reduced activation and oxidation of the antioxidant peroxiredoxin-1 (prdx1) and reduced p53 expression and carcinogenesis in the mammary gland. These results provide a compelling rationale to explore marginal Zn intake as a modifiable factor that reduces the protective effect of breastfeeding on carcinogenesis in women.

Materials and methods

The present study was approved by the IACUC at the Pennsylvania State University which is accredited by the American Association for the Accreditation of Laboratory Animal Care.

DMBA tumor model validation. We first validated our carcinogenesis model in mice. Female C57BL/6 mice were obtained commercially (Charles River Laboratories, Wilmington, MA, USA) and housed in polycarbonate cages. Mice (n=36) were fed a commercially available, casein based, purified diet based on AIN93 (MP Biomedicals, Solon, OH, USA) containing 30 mg Zn/kg. One cohort of mice (n=18) remained nulliparous and the other cohort (n=18) was bred and allowed to deliver offspring naturally. One litter was not viable and the mouse

was excluded from further analysis. The offspring were naturally weaned and removed from the dams entirely on lactation day 21. Two weeks post-weaning, the mice from both cohorts were gavaged with 7,12-dimethylbenz(a)anthracene (DMBA, 1 mg in corn oil) weekly for 4 weeks. Beginning at 5 weeks following the final dose DMBA, the mammary glands were palpated weekly and tumor size was measured using digital calipers. Mice were euthanized by CO_2 asphyxiation after 24 weeks, if body weight declined >10% or if the tumor size was ≥15 mm. These data were used for Kaplan-Meir analysis to validate our mouse model.

Zinc deficiency model
Animals. Next, female C57BL/6 mice were obtained commercially (Charles River Laboratories) and housed in wire bottom polycarbonate cages. Mice (n=30/diet) were fed a commercially available, casein based, purified diet based on AIN93 (MP Biomedicals) containing adequate Zn (30 mg Zn/kg; ZA) or a marginal reduction in Zn (15 mg Zn/kg; ZD) for 30 days. Zn concentration was confirmed by atomic absorption spectrophotometry. Mice were bred and allowed to deliver offspring naturally. Mice that did not give birth (ZA, n=5; ZD, n=4) or did not nurse their pups following delivery (ZA, n=3; ZD, n=2) were excluded from analysis. Mice were maintained on their respective experimental diets throughout the study. Offspring were naturally weaned and removed from the dams entirely on lactation day 21. Two weeks post-weaning, dams were gavaged with either 0.1 ml corn oil (vehicle control; n=6) or DMBA (1 mg/0.1 ml corn oil; n=18) weekly for 4 weeks. Beginning at 5 weeks after the final dose of DMBA or corn oil, mice were weighed and examined and the mammary glands were palpated weekly. Mice were euthanized by CO_2 asphyxiation after 24 weeks, if the body weight declined >10% or if the tumor size was ≥15 mm. Blood, mammary glands and liver were collected and processed for biochemical and histological analysis as previously described (5). Tumors were removed, weighed and measured, then snap-frozen or fixed overnight in phosphate-buffered paraformaldehyde (4%), pH 7.4.

Histology. Fixed glands were washed three times for 30 min in phosphate-buffered saline (PBS) and three times for 30 min in 70% ethanol at 4˚C. The glands were embedded in paraffin and 5 μm sections were adhered to positively-charged glass slides. Hematoxylin and eosin (H&E), Trichrome staining and immunohistochemistry for 8-hydroxyguanosine (8OHdG) were performed as previously described (6). Sections were examined by light microscopy (Leica DM IL LED; Leica Microsystems GmbH, Wetzlar, Germany) and images were captured using Leica Application Suite (version 3.6.0) at x4, x10 and x40 magnification.

Tissue Zn measurement. Zn concentration in diet, plasma, liver, mammary gland and tumors was determined by atomic absorption spectrophotometry (AAnalyst 400; Perkin Elmer, Akron, OH, USA) as previously described (5).

Immunoblotting. Cell extracts and crude membrane protein homogenates were generated as previously described (5). Equal amounts (20-60 μg) of membrane proteins were diluted 1:1 in Laemmli sample buffer with DTT (100 mM) and resolved by

SDS-PAGE (10%) at 200 V for 1 h. Proteins were transferred to nitrocellulose membrane at 100 V for 1 h. The membrane was blocked in 1% BSA for 1 h at room temperature and then incubated simultaneously with either primary antibody (ZnT2, ZIP6; 1:1,000) and β-actin antibody as loading control (1:5,000) diluted in LI-COR buffer (LI-COR Biosciences, Lincoln, NE, USA) overnight at 4°C. Membranes were washed three times for 5 min in PBS with 0.1% Tween (PBS-T) and then incubated for 1 h at room temperature with infrared secondary antibody (IRDye® 800CW goat anti-rabbit IgG diluted 1:20,000 in LI-COR buffer). The membranes were washed three times for 5 min in PBS-T and rinsed in PBS before scanning on the Odyssey® CLX imaging system (LI-COR Biosciences). Protein quantification was performed using Odyssey Image Studio ver 2.0. Alternatively, membranes were blocked in 5% non-fat milk/PB-T for 1 h and incubated in 5% non-fat milk/PBS-T containing primary antibody (p53, 1:1,000; Santa Cruz Biotechnology, Dallas, TX, USA), washed three times in PBS-T and then incubated for 1 h at room temperature with donkey anti-rabbit IgG conjugated to horseradish peroxidase (1:30,000; GE Healthcare Life Sciences, Pittsburgh, PA, USA). The membranes were washed three times for 5 min in PBS-T and visualized with SuperSignal Femto (Thermo Fisher Scientific) and visualized using autoradiography film. Membranes were stripped and reprobed for β-actin as a loading control. For analysis of prdx1 and oxidized prdx1 (prdx1-SO$_3$), tissues were homogenized in Tris lysis buffer (50 mM Tris, 2% Triton X-100, 0.5 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 10% glycerol; 50 mM NaF, 1 mM NaVO$_4$, 40 mM β-glycerophosphate, 30 μg/ml catalase and proteinase inhibitors). Protein concentrations were quantified using the BCA protein assay kit, according to the manufacturer's instructions (Thermo Fisher Scientific). Proteins were boiled in Laemmli sample buffer (Bio-Rad Laboratories) in the presence or absence of β-mercaptoethanol (Sigma) for 10 min. Whole cell lysates were fractionated by SDS-PAGE, and transferred to a nitrocellulose blotting membrane, following the manufacturer's instructions. Membranes were blocked with 5% BSA in Tris buffered saline (TBS) for 30 min, and incubated with antibodies against prdx1 (1:4,000; Abcam), prdx1-SO$_3$ (1:500; Abcam), or β-actin (1:1,000; Oncogene), overnight at 4°C. Membranes were washed four times for 5 min in TBST (TBS containing 0.05% Tween-20), and visualized by infrared or chemiluminescent detection. For infrared processing, membranes were incubated with anti-goat IRDye, anti-rabbit IRDy®, or anti-mouse IRDye® (1:15,000; LI-COR Biosciences) for 30-45 min at room temperature. Blots were washed with TBST three times and with TBS once, and imaged on the Odyssey® CLX imaging system. Protein quantification was performed using Odyssey Image Studio ver 2.0. Alternatively, membranes processed by chemiluminescence were incubated HRP-conjugated anti-mouse or anti-rabbit antibodies (1:10,000) for 1-1.5 h at room temperature. Blots were washed four times with TBST for 5 min, and exposed to enhanced chemiluminescence substrate (ECL; Thermo Fisher Scientific) for 1 min and visualized using autoradiography film.

Statistical analysis
Tumor model. Statistical analysis was performed to determine whether DMBA-exposed mice fed ZD developed significantly
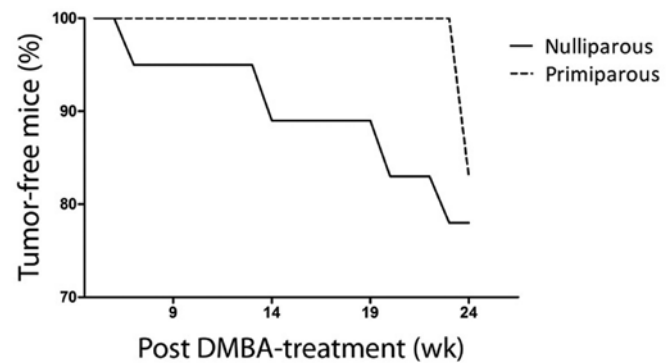

Figure 1. Lactation protects against breast cancer in a DMBA-induced tumor model in mice. Kaplan-Meier analysis indicated that there was a significant protective effect of lactation on carcinogenesis in our mouse model (P<0.0001).

more mammary gland tumors with shorter latency to tumor formation and higher mortality than DMBA-exposed mice fed ZA. Due to small sample size and non-parametric distribution of the data, univariate analysis using Fisher's exact test for binary variables and Mann-Whitney U test for continuous variables was performed. All statistical analyses were performed using STATA (version 12.1; StataCorp LLP, College Station, TX, USA). Statistical significance was demonstrated at P<0.05.

Tissue analysis. All other data are presented as means ± standard deviation. Statistical comparisons were performed using Student's t-test or a one-way ANOVA where noted (GraphPad Prism, Berkeley, CA, USA) and a significant difference was demonstrated at P<0.05.

Results

Model validation: lactation protects against carcinogenesis in DMBA-exposed mice. We first conducted a pilot study in mice to verify that gavage administration of DMBA produced mammary tumors, and that lactation protected against carcinogenesis as previously described in rats (29). The ability of DMBA to generate tumors in nulliparous mice was compared with mice that had successfully completed lactation. Fig. 1 summarizes tumor development over the entire 24 weeks of the study in nulliparous mice, compared to the appearance of tumors only at the very end of the study period in primiparous mice that completed one pregnancy-lactation cycle (P<0.0001).

Marginal Zn intake throughout one pregnancy-lactation cycle leads to a toxic microenvironment in the mammary gland. Similar to our previous studies in male (30), nulliparous (6) or lactating mice (5), long-term feeding of a marginal Zn diet throughout a complete pregnancy-lactation cycle in female mice did not significantly affect the Zn concentration in plasma (ZA, 11.04±2.83 μM; ZD, 9.92±0.37 μM) or mammary gland (ZA, 5.5±3.4 μg/g tissue; ZD, 6.4±3.4 μg/g tissue). This confirms that mice maintained on a marginal Zn diet are not severely Zn deficient. Additionally, there was no effect of marginal Zn intake on body weight gain over the course of the study (ZA, 3.8±1.6 g; ZD, 4.6±1.3 g), further verifying the marginal dietary insult.
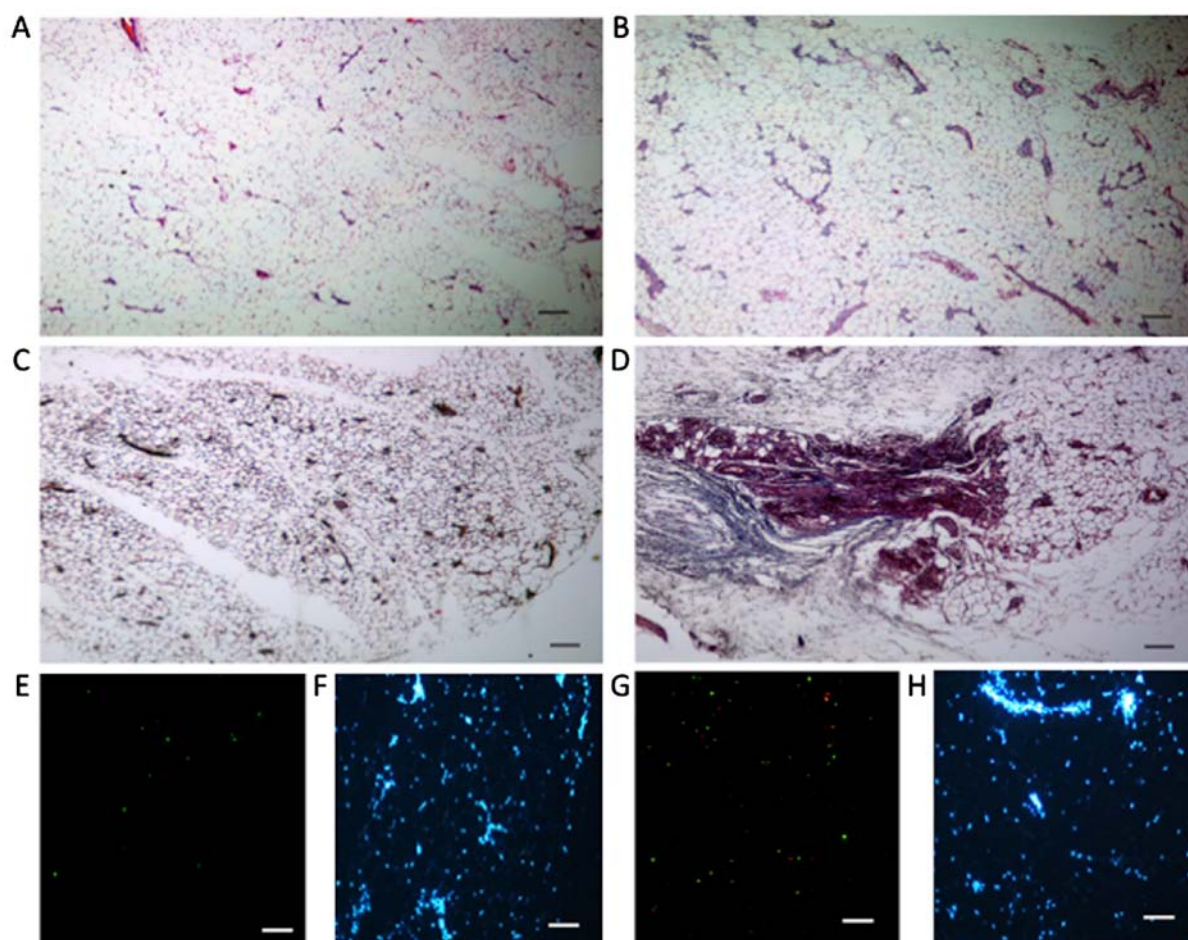
Figure 2. Mammary gland involution was impaired in mice fed a marginal Zn diet. H&E-stained sections of a mammary gland from mice fed a control (ZA; A) or marginal Zn (ZD; B) diet. Note the excess ductal and alveolar structures in mice fed a ZD diet. Trichrome stained sections illustrating accumulation of collagen (blue) mammary gland from mice fed a ZA (C) or ZD (D) diet. Mammary gland sections stained for 8OHdG (green) in mice fed a ZA (E) or ZD (G) diet. Sections were counter-stained with DAPI (ZA, F; ZD, H) Magnification, x10; scale bar, 50 μm.

We previously reported that marginal Zn intake in nulliparous mice increases oxidative stress, inflammation and collagen deposition in the mammary gland, paradoxically creating a toxic mammary gland microenvironment that led to disrupted mammary gland development (6). In the present study, we determined that a complete pregnancy-lactation cycle did not resolve this toxic microenvironment. Moreover, H&E staining of mammary gland sections indicated that mice fed ZD retained more ductal and alveolar structures following involution compared with mice fed ZA (Fig. 2A and B). Similar to what we observed in nulliparous mice, trichrome staining indicated that the mammary glands of mice fed ZD were enriched in collagen (Fig. 2C and D). Finally, we found that mice fed ZD had significantly more cells with 8OHdG-stained nuclei (31±1.4 positive-stained nuclei) compared with mice fed a ZA diet (13.7±2.5, P<0.05; Fig. 2E-H). Collectively, this suggests that the toxic microenvironment in the mammary glands of mice fed ZD was retained following lactation, and importantly, interfered with mammary gland remodeling following weaning.

Marginal Zn intake reduces the protective effect of lactation on carcinogenesis. We next assessed effects of marginal Zn intake on DMBA-initiated carcinogenesis after a complete pregnancy-lactation cycle. We found that 11.8% of DMBA-exposed mice fed a ZA diet had palpable mammary tumors. In contrast, 35% of mice fed ZD had palpable mammary tumors. While this did not reach statistical significance (P=0.137), the number of ZD mice with palpable tumors was similar to the number of nulliparous mice with palpable mammary tumors (50%). This effect was not mammary gland-specific as we noted the incidence of total tumors in DMBA-exposed mice fed ZD was significantly higher (70%) compared with DMBA-exposed mice fed ZA (29.4%; P<0.05). Following euthanization and dissection, we noted that non-palpable tumors were present in many mice. Histological examination determined that only 1 mouse (7%) fed ZA had a non-palpable tumor, while 6 mice (38%) fed ZD had non-palpable tumors (P=0.086). Because only one mouse fed ZA had a non-palpable tumor, we were unable to do any further comparison. In addition, DMBA-exposed mice fed ZD tended to have a shorter latency to tumor formation, although this comparison did not reach statistical significance (log rank, P=0.0846). Notably, mice fed ZD and exposed to DMBA had significantly greater mortality (40%) compared with mice fed ZA (6%, log rank, P=0.0175). Collectively, these observations suggest that marginal Zn intake may reduce the protective effect of lactation on carcinogenesis and enhances mortality.
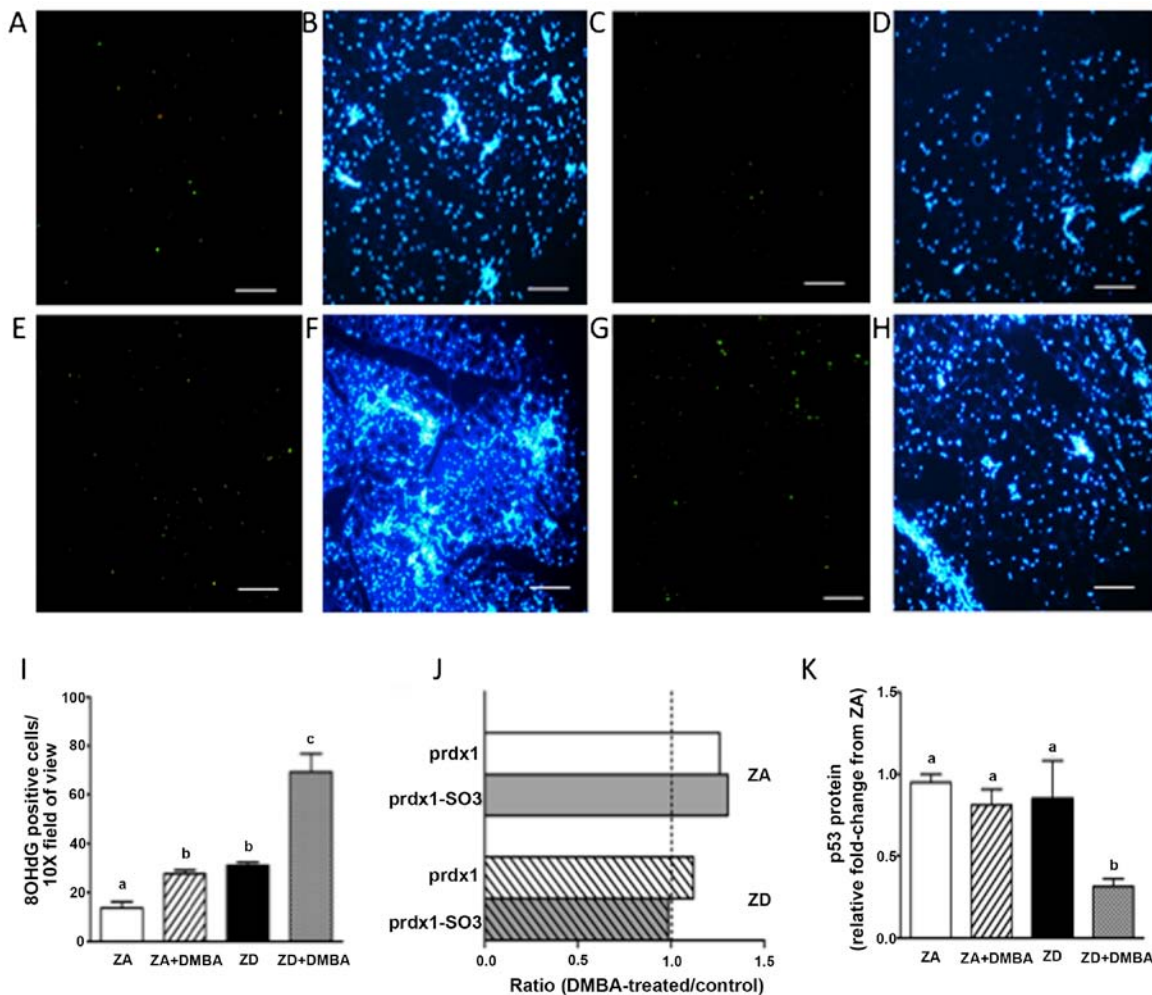
Figure 3. A marginal Zn diet increases oxidative stress which was augmented by DMBA exposure. Mammary gland sections stained for 8OHdG (green) in mice fed a control (ZA; A and E) or a marginal Zn (ZD; C and G) diet and treated with DMBA (E and G). Sections were counterstained with DAPI (ZA: B and F; ZD: D and H). Magnification, x10; scale bar, 50 μm. (I) Data represent mean 8OHdG stained cells/10X field of view ± SD, n=5 mice/group. Means with different letters are significantly different; P<0.05. (J) Data represent mean ratio of DMBA-treated/control mice for both the antioxidant peroxiredoxin 1 (prdx1) and oxidized prdx1 (prdx1-SO$_3$) in mice fed ZA and ZD, n=6 mice/group. Note a ratio of 1.0 (dashed line) indicates no increase in prdx1 or oxidized prdx1 in response to DMBA. (K) Data represent mean p53 protein relative to β-actin ± SD, n=6 mice/group. Means with different letters are significantly different, P<0.05.

Marginal Zn intake augments DMBA-induced oxidative stress, resulting in a paradoxical Zn accumulation in breast tissue, associated with reduced p53 expression, ductal hyperplasia and enhanced tumor formation. To address the biological underpinnings, we first assessed Zn status. As noted above, a marginal Zn diet did not affect plasma Zn concentration. In contrast, DMBA-exposure in mice fed the ZD diet led to significantly lower plasma Zn levels (9.1±1.6 μM) compared to mice fed the ZA diet (11.1±1.7 μM, P<0.001). As noted above, mammary gland Zn concentration in mice fed the ZD diet was not different from those fed the ZA diet. In contrast, DMBA-exposure in mice fed ZD led to a greater degree of Zn accumulation in the mammary gland (3.3±2.0 μg/g tissue) compared to ZA mice (2.1±1.4 μg/g tissue); however, this did not reach statistical significance (P=0.07). In addition, we noted that mammary gland Zn concentration in mice fed ZD and exposed to DMBA was highly variable (1.11-8.26 μg Zn/g tissue). We found that mammary tissue that contained non-palpable tumors had a significantly greater Zn concentration (4.9±2.4 μg Zn/g) compared with mammary tissue that did not

have non-palpable tumors (2.6±1.4 μg Zn/g, P<0.05), consistent with reports that breast tumors accumulate Zn. Because only one mouse fed a ZA diet had a non-palpable tumor, a relationship between tissue Zn concentration and the presence of non-palpable tumors could not be evaluated. Additionally, we noted that in mice fed ZD and exposed to DMBA, there was a significant inverse correlation between plasma and mammary gland Zn concentrations (P<0.05).

To evaluate oxidative stress in mammary tissues, we measured the number of cells with 8OHdG$^+$ nuclei (Fig. 3A-I). Few 8OHdG$^+$ nuclei were detected in mice fed the ZA diet (Fig. 3A and B) and 8OHdG$^+$ nuclei were most frequently detected in mice fed a ZD diet and exposed to DMBA (Fig. 3G and H). Intermediate densities of 8OHdG$^+$ nuclei were detected in mice fed a ZD diet (Fig. 3C and D) and mice fed the ZA diet and exposed to DMBA (Fig. 3E and F). In addition, mice fed a ZA diet had a modest increase in abundance of the antioxidant prdx1 in response to DMBA, which was monitored by the ratio of oxidized prdx1/reduced prdx1 (Fig. 3J). In contrast, this effect was not observed in ZD mice exposed to DMBA. These
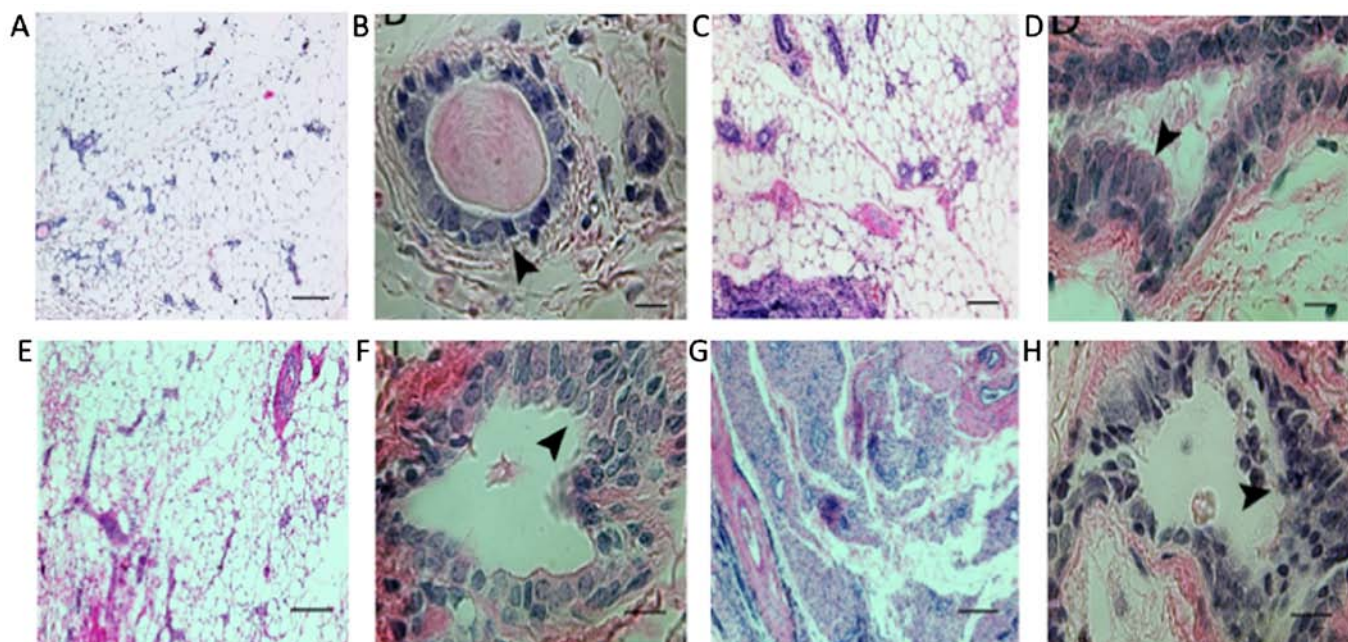
Figure 4. DMBA-exposure in marginally Zn deficient mice led to atypical ductal hyperplasia. H&E stained sections of mammary gland from mice fed a control (ZA, A and B) or a marginal Zn (ZD, C and D) diet and then exposed to DMBA (ZA, E and F; ZD, G and H). Magnification, x10; scale bar, 50 μm (A, C, E and G). Magnification, x40; scale bar, 10 μm (B, D, F and H). Note the layers and disorganization of ductal epithelial cells denoted by the arrowhead illustrating a single, well-organized ductal layer in ZA mice (B), a bilayer of ductal cells in ZD mice (D), ductal hyperplasia (3-4 cell layers) in ZA mice exposed to DMBA (F), and atypical hyperplasia (multiple, disorganized cell layers) in ZD mice exposed to DMBA (H).
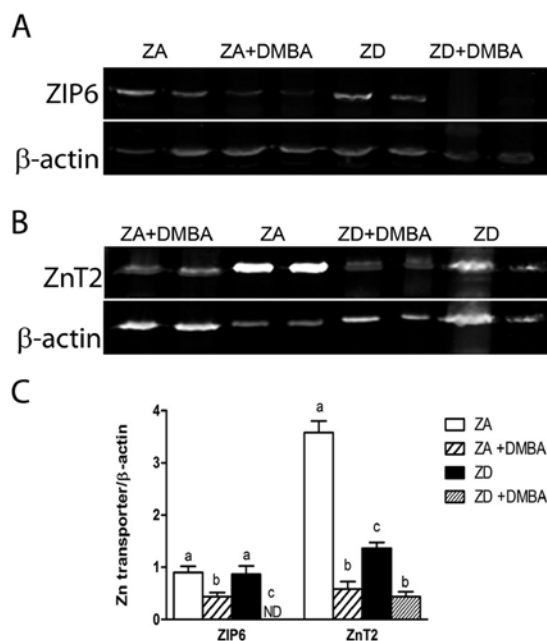

Figure 5. ZIP6 and ZnT2 abundance are reduced in response to DMBA. (A) Immunoblot of ZIP6 in total membrane fractions isolated from mammary glands of mice fed a control (ZA) or a marginal Zn (ZD) diet and exposed to DMBA (+DMBA). β-actin served as a loading control. (B) Representative immunoblot of ZnT2 in total membrane fractions isolated from mammary glands of mice fed ZA, ZD and exposed to DMBA (ZA+DMBA or ZD+DMBA). β-actin served as a loading control. (C) Data represent mean ratio of Zn transporter/β-actin from two independent experiments, n=4 mice/group. Means with different letters are significantly different; P<0.05.

observations suggest a reduced capacity to respond to oxidative stress in mice fed a ZD diet. Finally, enhanced oxidative stress

in mammary tissue of mice fed ZD and exposed to DMBA was associated with significantly less expression of the tumor suppressor p53 (Fig. 3K). Importantly, oxidative stress was associated with ductal hyperplasia (Fig. 4). Mice fed ZA had the expected patern, well-formed ducts with 2 well-organized cell layers (luminal and myoepithelial; Fig. 4A and B). When ZA mice were exposed to DMBA, ductal structures were less well-formed and contained 2-3 cell layers and contained columnar-shaped cells (Fig. 4C and D). However, when mice were fed ZD, the ductal structures were more disorganized and hyperplastic containing 3-4 cell layers (Fig. 4E and F), while examination of ductal structures in mice fed ZD that were exposed to DMBA revealed profoundly disorganized, hyperplastic and atypical cells (Fig. 4G and H), consistent with decreased p53.

Non-palpable tumors in mice fed a marginal Zn diet has lower expression of ZIP6 and ZnT2. To determine if key Zn transporters that have been associated with breast cancer were affected in our model, we assessed expression of ZIP6 and ZnT2. Previous reports found that ZIP6 expression is associated with better breast cancer prognosis (27). We detected robust expression of ZIP6 in mice fed both ZA and ZD; however, DMBA-treatment either reduced (in mice fed ZA) or completely eliminated (in mice fed ZD) ZIP6 expression in the mammary gland (Fig. 5A and C). In addition, we have previously reported that ZnT2 overexpression protects breast tumor cells from the cytotoxic effects of Zn hyper-accumulation (13), and that overexpression of ZnT2 in invasive MDA-MB-453 cells reduces invasion *in vitro* (23). In the present study, we detected robust expression of ZnT2 in mice fed ZA; however, ZnT2 abundance was reduced ~3-fold in mice fed ZD and

almost eliminated in mice exposed to DMBA, regardless of Zn intake (Fig. 5B and C). These observations are consistent with the hypothesis that loss of ZIP6 and ZnT2 expression may increase susceptibility of mammary epithelial cells to carcinogenesis.

Discussion

The present study suggests a potential relationship between consuming a marginal Zn diet and loss of the protective effect of lactation on carcinogenesis. This finding is particularly relevant to women's health as 60-80% of reproductive age women do not consume adequate Zn (7-9). Most animal models that explore effects of Zn intake utilize a severe dietary Zn restriction paradigm (<1 mg Zn/kg diet), which significantly reduces serum Zn concentration and causes profound physiologic dysfunction. It is possible that a more profound Zn restriction would have caused a more pronounced effect on carcinogenesis. However, our model more closely reflects the more marginal reduction in Zn intake that occurs in humans, which still led to a toxic mammary gland microenvironment including enhanced oxidative stress, inflammation and fibrosis. Importantly, this toxic microenvironment was associated with insufficient tissue remodeling following post-lactational involution. Moreover, in response to a subsequent carcinogenic insult, tissue Zn level and oxidative stress were augmented, and mice fed a marginal Zn diet were not able to respond rigorously to oxidative stress, which was associated with a modest decrease in tumor latency and potentially more invasive phenotype. This supports the need to better understand effects of marginal Zn intake on carcinogenesis in women.

Lactation protects against carcinogenesis from 3 different perspectives (31); through the elimination of pre-malignant cells during the extensive remodeling that occurs post-lactation, by the decreased life-time exposure to estrogen signaling, or through restricted proliferation and increased differentiation of breast cells that occurs during lactation. Although we previously found that marginal Zn intake in mice increased expression of ERα in the mammary gland (6), we were not able to find a consistent effect on ERα in the present study (data not shown). Further well-controlled studies in ovariectomized mice are required to determine if the effects of marginal Zn intake on carcinogenesis are executed through effects on estrogen and/or ER signaling in the mammary gland. However, we previously showed that marginal Zn intake substantially reduces the degree of differentiated epithelium and mammary gland activity during lactation (5), and now show that mammary gland remodeling is also compromised. This suggests that adequate tissue remodeling, beyond simply the removal of pre-malignant cells, may play a role in protection from breast cancer and that marginal Zn intake compromised the combined protection afforded by a complete pregnancy-lactation cycle. The mechanisms responsible for these profound effects are not currently understood but may involve defects in mammary gland expansion and remodeling through alterations in Zn-dependent matrix metalloproteinase activity (6) or reduced Jak2/Stat5 signaling (32). A major finding from the present study was that marginal Zn intake led to the loss of ZnT2 and the retention of ductal and alveolar structures following involution. This is consistent with the critical role that ZnT2-mediated Zn transport plays in regulating mammary gland involution that has recently been reported (33).

One key question is why poor remodeling might leave one more susceptible to carcinogenesis? Previous studies suggest that retention of differentiated ductal structures may increase tissue susceptibility to carcinogenic insults (34). In addition, marginal Zn intake led to enhancement of oxidative stress, which provides a major carcinogenic insult that drives the initial stages of disease. Reactive oxygen species damage DNA through oxidation, methylation, de-amination and de-purination and disrupt the system of oxidative damage repair through oxidation of their catalytic moieties. In fact, a recent report from Patel and colleagues (35) found that breast tissue enriched in oxidative stress had substantial differences in their 'biochemical fingerprint' suggesting profound changes in cell metabolism prior to (or perhaps during) carcinogenesis. Notably, while DMBA increased oxidative stress in the mammary glands of Zn adequate mice, the mice were able to activate the antioxidant prdx1 suggesting that these mice were able to manage the oxidative stress caused by the carcinogen. In contrast, in response to marginal Zn intake, oxidative stress was augmented by DMBA administration and was associated with more tumors and tumors that were more invasive. This suggests that marginal Zn intake compromises the ability to manage oxidative stress through reduced activity of key Zn-dependent enzymes such as prdx1 or Cu, Zn superoxide dismutase (36). In addition, both oxidative stress and collagen deposition stimulate macrophage infiltration which may perpetuate the inflammatory microenvironment. Importantly, collagen deposition results in higher breast density, and contributes to mammary tumor formation and metastasis (37) making it a predictive risk factor for breast cancer in women.

Consistent with previous reports in human breast tumors (12) we found that malignant tissue accumulated Zn. Interest in better understanding mechanisms responsible for paradoxical Zn accumulation in marginally Zn deficient mice led us to assess the abundance of ZIP6 and ZnT2, as over-expression of both have been noted in breast cancer. However, we found that both ZIP6 and ZnT2 expression were substantially reduced by DMBA treatment in both Zn adequate and marginally Zn deficient mice. This suggests that ZIP6 is not responsible for Zn accumulation in tumors in this model and thus Zn accumulation is likely driven by increased expression of other ZIP proteins (14). Moreover, the reduction in ZnT2 suggests that excess Zn is not sequestered into vesicles to protect the cells from cytotoxicity which may underlie some of the observations such as enhanced oxidative stress in this study. The loss of ZnT2 is important as we previously showed that ZnT2 expression is inversely related to invasion (23). The mechanisms through which ZIP6 and ZnT2 are reduced are not understood, but may affected either directly or indirectly by oxidative stress (38).

In conclusion, marginal Zn intake in mice created a toxic microenvironment in the mammary gland including enhanced oxidative stress, inflammation and mammary gland fibrosis that was associated with insufficient tissue remodeling following lactation, and led to a modest reduction in tumor latency and a more-invasive tumor phenotype. Collectively, the present study implicates Zn intake as an important dietary

modifier of the protective effect of lactation on carcinogenesis and may provide insight into the variability of the protective effect of breastfeeding on breast cancer.

Acknowledgements

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