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Manganese superoxide dismutase is dispensable for post-natal development and lactation in the murine mammary gland

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Abstract

Mammary gland development is a multistage process requiring tightly regulated spatial and temporal signalling pathways. Many of these pathways have been shown to be sensitive to oxidative stress. Understanding that the loss of manganese superoxide dismutase (*Sod2*) leads to increased cellular oxidative stress, and that the loss or silencing of this enzyme has been implicated in numerous pathologies including those of the mammary gland, we sought to examine the role of *Sod2* in mammary gland development and function *in situ* in the mouse mammary gland. Using Cre-recombination driven by the mouse mammary tumor virus (MMTV) promoter, we created a mammary-specific post-natal conditional *Sod2* knock-out mouse model. Surprisingly, while substantial decreases in *Sod2* were noted throughout both virgin and lactating adult mammary glands, no significant changes in developmental structures either pre- or post-pregnancy were observed histologically. Moreover, mothers lacking mammary gland expression of *Sod2* were able to sustain equal numbers of litters, equal pups per litter, and equal pup weights as were control animals. Overall, our results demonstrate that loss of *Sod2* expression is not universally toxic to all cell types and that excess mitochondrial superoxide can apparently be tolerated during the development and function of post-natal mammary glands.

Keywords

mouse mammary tumor virus; breast; mouse; reactive oxygen species; milk

Introduction

Mammalian mammary glands are derived from the ventral ectoderm, and development of these organs occurs in four distinct stages: embryonic, neonatal, pubertal, and reproductive [1,2]. In fully differentiated virgin mammary glands, epithelial cells line the lumens of ductal structures while myoepithelial cells surround these arrangements [3]. During lactation, milk producing alveoli derive from the epithelial ductal cells, thus creating a third major cell type within the mammary gland [3]. Each stage of development is characterised

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Declaration of interest

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Author contributions

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by complex and highly regulated signalling pathways (e.g. Jak/Stat, estrogen signalling, Jnk/Erk, etc.) required for proper development [4,5]. Interestingly, many of the proteins and transcription factors necessary for mammary gland development have demonstrated differential sensitivity to oxidative stress [6–9]. Furthermore, various studies have verified the effects of increased oxidative stress on fully differentiated mammary tissue and mammary carcinoma [10,11]. In contrast, while exhaustive *in vitro* examinations have shown reactive oxygen species (ROS) may induce aberrant protein and transcription factor signalling in various adult mammary tissue models [12–14], limited *in situ* data have demonstrated the role of oxidative stress in the mammalian mammary gland through its full developmental cycle.

Due to the respiratory function of the mitochondria during aerobic metabolism, this organelle serves as a major source of ROS in eukaryotic cells [15]. Additionally, non-pathologic mammary glands are rarely hypoxic, and primarily rely upon aerobic metabolism for ATP production [16]. Normally, the mitochondrially located antioxidant enzyme manganese superoxide dismutase (*Sod2*) maintains a nominal steady-state level of superoxide [17]. The loss or silencing of *Sod2* has demonstrated increased cellular oxidative stress, developmental aberrations, and exacerbated carcinogenesis in various experimental models [18–22]. With this understanding, it is hypothesised that *Sod2* plays a critical role in the development and function of normal mammalian mammary tissue. To date, only one study has demonstrated aberrant embryonic development of the mammary gland in the absence of *Sod2* [23]. Using murine *Sod2* deficient mammary anlagen implanted into the renal capsule of recipient mice, it was determined that the absence of *Sod2* caused decreased E-cadherin expression, increased Ki-67 labelling, and hyperplasia of the explanted mammary gland. While these results confirm *Sod2* dependence in embryonic mammary development, the role of *Sod2* in later stages of development remains unclear. To address these deficits in our knowledge, we employed a mammary-specific *Sod2* conditional knock-out mouse to examine the effects of increased oxidative stress in post-natal mammary gland growth and function.

Methods

Mice and characterisation

Mice homozygous for the floxed *Sod2* allele (i.e. B6.Cg-*Sod2*^{tm1}, shorthand *Sod2*^{L/L}), in which exon 3 of the *Sod2* gene is flanked by 2 loxP sequences, have been previously described [24]. B6129-Tg(MMTV-cre)^{4Mam/J} or MMTV-Cre mice (cre-recombinase is exogenously expressed under control of the mouse mammary tumour virus (MMTV) LTR where Cre-recombinase expression is primarily limited to mammary epithelial cells in virgin and lactating mice) were purchased from Jackson Laboratories, and have been previously described [25]. To obtain conditional mammary *Sod2* homozygous knock-out animals (i.e. *Sod2*^{-/-}), parent strains of both the floxed *Sod2* and MMTV-Cre mice were bred to the F2 generation. MMTV-Cre was only passed through male parents to limit non-specific oocyte expression. Due to the variability in strains, littermate floxed animals (i.e. *Sod2*^{L/L}) served as controls. Genotyping, real-time quantitative RT-PCR, and western blotting were performed as previously described [18,26]. Oligonucleotides used for real-time quantitative RT-PCR are as follows: Forward *Sod2*: GCTCTGGCCAAGGGAGATGT; Reverse *Sod2*: GGGCTCAGGTTTGTCCAGAAA; Forward 18s: GCCCGAAGCGTTTACTTTGA; Reverse 18s: TCATGGCCTCAGTTCCGAA; Forward *Sod1*: AATGTGACTGCTGGAAAGGACG; Reverse *Sod1*: GACCACCATTGTACGGCCAA; Forward *Cat*: GCTCAGCCCTGGAGCACAG; Reverse *Cat*: CTTTGTGTAGAATGTCCGCACCT; Forward *Gpx1*: TTTCCCGTGCAATCAGTTC; Reverse *Gpx1*: TCGGACGTACTTGAGGGAAT. Antibodies used are as follows: *Sod2* (Catalog #06-984, Millipore, USA), Beta-actin (Catalog #E7, University of Iowa Hybridoma

Core, Iowa City, IA), and 8-oxo-2'-deoxyguanosine (Catalog #MAB6116, Abnova, Walnut, CA). For all experiments, fresh tissue was harvested and used from female animals at 8–10 weeks of age, unless otherwise noted. All work was performed under the approval of the Institutional Animal Care and Use Committee at the University of Iowa.

Mammary whole mounts and histology

For whole mounts, inguinal mammary glands were excised from virgin mice and fixed overnight on glass slides in 75% EtOH + 25% Acetic Anhydride (v/v). Following this, slides were placed in 70% EtOH followed by distilled water, and then allowed to incubate in haematoxylin (SigmaPath) until fully stained. Whole mounts were cleared using acid alcohol (0.25% HCl in EtOH), and blued using lithium carbonate (1.36%). Finally, slides were dehydrated in increasing concentrations of EtOH, cleared using xylenes, and stored in light mineral oil for long-term storage. For histology, tissues were fixed in 10% neutral formalin, then processed and embedded in paraffin. Four- to five-micrometer sections were prepared with a microtome (HM 355, Microm, Walldorf, Germany) and then stained with hematoxylin and eosin (H&E) with an automated slide stainer (Sakura DRS 601 Diversified Stainer, Sakura, Hayward, CA). Stained slides were mounted with Solvent 100 mounting media, and brightfield micrographs of stained sections were taken with a microscope (Olympus BX-51, Olympus, Melville, NY) fitted with an Olympus DP70 camera (Olympus).

8-oxo-2'-deoxyguanosine immunohistochemistry

Mouse mammary tissues were sectioned (4 μ m) and hydrated through a series of ethanol and xylene baths. Antigen retrieval was performed with citrate buffer (pH 6.0) in a commercial heat-induced epitope unit according to manufacturer's instructions (125°C $\times$ 5 minutes, Decloaker ChamberTM NxGen, Biocare Medical, Concord, CA, USA). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (8 minutes). Non-specific antibody binding Fc receptors (60 minutes $\times$ 10% solution, HB-197TM, American Type Culture Collection (ATCC), Manassas, VA, USA) and general tissue blocking (60 minutes, Background Buster, Innovex Biosciences, Richmond, CA) were performed to prevent background staining. Primary mouse monoclonal anti-8-oxo-2'-deoxyguanosine was applied (1:100 $\times$ 60 minutes) and after washes, a secondary Ab kit was applied according to manufacturer's instructions (Dako Mouse Envision, Dako, Carpinteria, CA). Slides were counterstained in hematoxylin (1 minutes), blued in Scott's tap water and routinely dehydrated through serial ethanol and xylene baths and coverslipped.

Statistics

Data were expressed as mean and standard deviation. All experiments were performed using 3 mice per group unless otherwise stated. For most experiments, comparisons between groups were analysed by unpaired 2-tailed Student's *t*-test. A *p*-value of less than 0.01 was considered to be significant.

Results

Sod2 is conditionally deleted in virgin and lactating mammary glands

We and others have demonstrated the conditional *Sod2* knock-out mouse to be a viable model of increased oxidative stress *in vivo* [18,27,28]. To understand the effects of increased oxidative stress on post-natal mammary gland development, we created a conditional mammary *Sod2* knock-out mouse using Cre-recombination driven under the control of the MMTV promoter. The MMTV promoter becomes activated shortly after birth (neonatal developmental stage), and has been demonstrated to be highly expressed in

epithelial and myoepithelial cells in both virgin and lactating mammary glands with little or no expression in stroma, fibroblasts, or adipocytes [29], making it a never before utilised model for studying *Sod2* loss in multiple cell types and stages of mammary gland development.

After breeding mice to the F2 generation, PCR analysis of tail sample DNA was used to confirm the proper genotype of animals (data not shown). Quantitative real-time RT-PCR analysis demonstrated an approximate 90% decrease in *Sod2* mRNA in mammary glands from both *Sod2*^{-/-} virgin and lactating mice compared to their *Sod2*^{+/+} littermate controls (Figure 1A). This loss of *Sod2* was also seen at the protein level as revealed by both western blot and immunohistochemical analysis in lactating mammary glands (Figure 1B, D). No off-target tissues queried demonstrated any significant decreases in *Sod2* mRNA (Figure 1C) supporting previous findings using MMTV driven Cre-recombination [25]. Overall, the MMTV driven Cre-recombinase-mediated excision of *Sod2* provides an optimal model for studying the role of this anti-oxidant enzyme in post-natal mammary gland development and function.

Increased oxidative stress does not alter other major antioxidant enzymes

To assess the level of oxidative stress in *Sod2*^{+/+} and *Sod2*^{-/-} mammary glands, immunohistochemical staining for 8-oxo-2'-deoxyguanosine was performed on lactating mammary gland tissue. Significant increases in 8-oxo-2'-deoxyguanosine staining are noted in the *Sod2*^{-/-} mammary glands when compared to *Sod2*^{+/+} controls (Figure 2A), thus confirming increased cellular oxidative stress due to the loss of *Sod2*. We additionally performed immunohistochemical analysis for 3-nitrotyrosine, but levels were undetectable in *Sod2*^{+/+} or *Sod2*^{-/-} mammary glands. This finding of undetectable levels of 3-nitrotyrosine is consistent with previous findings using the *Sod2* conditional mouse [18]. Furthermore, it would be hypothesised that this cellular oxidative stress may elicit compensatory changes in other major cellular antioxidant enzymes. To address this, quantitative real-time RT-PCR analysis was performed for Cu/Zn SOD (*Sod1*), catalase (*Cat*), and glutathione peroxidase 1 (*Gpx1*; Figure 2B). Surprisingly, no significant changes were noted in any of the antioxidant enzyme mRNA levels suggesting other mechanisms for adaptation to increased oxidative stress may be in effect.

The loss of *Sod2* has no apparent effect on post-natal mammary development

To understand the consequence of *Sod2* loss in mammary development, whole mounts of 8–10 week old virgin mammary glands were prepared (Figure 3A). No significant differences were noted between *Sod2*^{+/+} and *Sod2*^{-/-} whole mounts, as similar quantities and qualities of ducts, branches and buds were observed in both groups. Histological analysis of virgin mammary glands also demonstrated no notable differences, as the number, size and quality of mammary epithelial structures were not significantly changed in *Sod2*^{-/-} versus *Sod2*^{+/+} (Figure 3B). Taken together, the data suggest that *Sod2* does not play a significant role in the development of pubertal mammary gland structures.

While *Sod2* loss did not hinder pubertal mammary development, the role of *Sod2* in the reproductive cycle of the mammary gland remained unknown. To address this, *Sod2*^{+/+} and *Sod2*^{-/-} female mice were mated with wild-type C57BL/6 males to induce mammary development and lactogenic differentiation. Seven days after birth (during peak lactation), mammary glands were extracted from the females and histologically analysed. Interestingly, no differences were observed between *Sod2*^{+/+} and *Sod2*^{-/-} lactating mammary glands as both demonstrated similar numbers and structures of differentiated ductal structures as well as colloidal material within the ducts (Figure 4A). Finally, mammary glands were extracted 14 days after weaning of the mice to assess differences during involution. Similar to the

previous stages of development, no significant differences were noted in the involuted mammary glands in the presence or absence of *Sod2* (Figure 4B). Taken together, these data imply that *Sod2* plays a minimal role in many or most stages of post-natal mammary gland development.

***Sod2* deficient mice lactate normally and sustain healthy litters**

While the physical structures comprising the mammary glands were shown to be unaffected by the loss of *Sod2*, the question still remained as to the functionality of the glands. To address this, *Sod2*^{L/L} and *Sod2*^{-/-} females were allowed to breed repetitively to assess the number of litters each group could sustain throughout their reproductive lifetime. Both *Sod2*^{L/L} and *Sod2*^{-/-} mothers were able to sustain equal numbers of pups per litter (Figure 5A) as well as a similar numbers of total litters (*Sod2*^{L/L} average = 8.4 ± 1.1 litters, *Sod2*^{-/-} average = 8.0 ± 1.6 litters, $p = 0.82$). In addition, the pups weights were unaffected by the loss of *Sod2* in the lactating mothers, suggesting the production of normal milk quantity and quality (Figure 5B).

It was hypothesised that after repetitive cycles of lactation and involution that a small population of cells may have escaped *Sod2* excision by poor Cre-recombinase penetrance and these cells may have selectively out-competed *Sod2*^{-/-} cells during the proliferation of the reproductive mammary gland. To address this, we performed quantitative real-time PCR analysis on lactating mammary glands from *Sod2*^{L/L} and *Sod2*^{-/-} females as a function of the numbers of litters they bore. It was determined that *Sod2* expression did in fact remain low regardless of litter number (Table I), indicating that the repopulation following involution was likely not accomplished by a stem cell compartment escaping *Sod2* excision. In summary, the data presented here strongly support a negligible role for *Sod2* in the normal development and function of murine mammary glands.

Discussion and conclusions

The results presented here demonstrate that normal mouse mammary gland development and function are minimally affected by the loss of *Sod2*. These results directly contrast with numerous reports of other organs systems that are highly dependent upon *Sod2* for proper development and survival. For example, the constitutive *Sod2* knock-out mouse is lethal shortly after birth due to severe systemic oxygen toxicity [21,22]. Furthermore, using the *Sod2* conditional knock-out mouse we and others have shown specific developmental aberrations to the loss of *Sod2* in tissues such as liver, muscle, neurons, and immune cells [18,27,28,30]. While we were unable to identify any compensatory up-regulation of other antioxidant enzymes (i.e. *Sod1*, *Cat*, *Gpx1*) in the *Sod2*^{-/-} mammary tissue, further examination is warranted in the elucidation of how this organ system is resistant to the effects of increased oxidative stress.

The use of MMTV-Cre recombination has been used extensively in the illumination of gene function necessary for normal mammary gland development and function [31–35]. While MMTV-Cre is presumably the best current model to study the effects of post-natal mammary growth, limitations exist within the model that may hinder the interpretation of results. MMTV-Cre recombination has been shown to be highly targeted to the epithelial and myoepithelial compartment of both virgin and lactating mammary tissue [25], but mosaicism of Cre-expression amongst the differentiated cell types of the mammary gland suggests a lack of full penetrance throughout the mammary gland stem cell population [29]. These findings imply that a small population of stem cells lacking Cre-recombination may in fact be able to drive mammary gland development and differentiation prior to the activation of MMTV and thus target gene excision. This phenomenon may explain the report of no effect of parathyroid hormone-related protein (PTHrP) deletion on mammary gland

development using MMTV-Cre recombination, when PTHrP has been shown to be critical to mammary gland growth in other models [36,37]. To address the deficits of the MMTV-Cre model, other models of mammary specific promoters of Cre-recombination could be used such as whey acidic protein, progesterone receptor or beta-lactoglobulin [25,38,39]. Each of the aforementioned models also possesses limitations on temporal and spatial expression of cre-recombinase, which confounds the ability to adequately study mammary gland development and function. While we acknowledge that the stem cells of our *Sod2*^{-/-} model may still retain *Sod2* function, using MMTV-driven Cre-recombination it was demonstrated that fully developed *Sod2*^{-/-} mammary glands did in fact lack greater than 90% *Sod2* mRNA and protein. These cells lacking *Sod2* revealed no changes in overall quality or quantity of cellular structures, and they were able to normally lactate to sustain equal numbers of healthy pups as the *Sod2*^{L/L} controls. Overall, while limitations of the MMTV-Cre model exist, the role of *Sod2* in mammary gland growth, development, and function appears insignificant.

In conclusion, we have used a novel model of targeted *Sod2* deletion to understand the role of *Sod2* in mammary gland development *in vivo*. While no quantifiable differences were observed between *Sod2*^{-/-} and *Sod2*^{L/L} animals, we believe these results contribute a significant finding to redox developmental biology. *Sod2* has repeatedly demonstrated a critical function to the development of mammalian organ systems, but we report for the first time a tissue apparently resistant to the loss of this anti-oxidant enzyme. Insight into the mechanism by which mammary tissue adapts or is resistant to amplified oxidative stress may increase our understanding of the tissue specific redox environment, and could lead to new targeted redox-based therapies in the future.

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Abbreviations

Sod2	manganese superoxide dismutase
MMTV	mouse mammary tumor virus
ROS	reactive oxygen species
<i>Sod2</i>^{L/L}	control loxP containing mouse
<i>Sod2</i>^{-/-}	conditional <i>Sod2</i> knock-out mouse
<i>Sod1</i>	copper/zinc superoxide dismutase
<i>Cat</i>	catalase
<i>Gpx1</i>	glutathione peroxidase 1
PTHrP	parathyroid hormone related protein

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Highlights

- Manganese superoxide dismutase (*Sod2*) has been implicated in mammary pathology
- We examine the role of *Sod2* in post-natal gland and development
- No structural differences were noted histologically in *Sod2* deficient mammary glands
- *Sod2* deficient mammary glands were able to lactate normally to sustain healthy pups
- We first report mammary tissue as resistant to increased oxidative stress by *Sod2* loss

Figure 1.

Sod2 is conditionally and effectively removed from virgin and lactating mammary tissue. (A) Quantitative real-time RT-PCR analysis of *Sod2* mRNA extracted from 8 to 10-week old *Sod2*^{L/L} (L/L) or *Sod2*^{-/-} (-/-) virgin or lactating mammary glands. Results normalised first to 18s mRNA then to respective *Sod2*^{L/L} by the ddCT method. (B) Western blot analysis of *Sod2* protein in 8–10-week old *Sod2*^{L/L} or *Sod2*^{-/-} lactating mammary glands. Beta-actin (ACTβ) is shown as a control for loading and transfer. (C) Quantitative real-time RT-PCR analysis of *Sod2* mRNA extracted from 8 to 10-week old *Sod2*^{L/L} (L/L) or *Sod2*^{-/-} (-/-) tissues. Results normalised first to 18s mRNA then to *Sod2*^{L/L} liver by the ddCT method. (D) Immunohistological analysis of *Sod2* protein in 8–10-week old *Sod2*^{L/L} or *Sod2*^{-/-} lactating mammary glands. Brown stain indicates *Sod2* immunoreactivity; blue indicates nuclear counterstain. Three mice per experiment were analysed; data are shown as mean and s.d. Where applicable, * = $p < 0.01$ by Student's *t*-test versus *Sod2*^{L/L}.

Figure 2.

Oxidative stress increases have no effect on major antioxidant enzymes. (A) Immunohistological analysis of 8-oxo-2'-deoxyguanosine in 8–10-week old *Sod2^{L/L}* or *Sod2^{-/-}* lactating mammary glands. Brown stain indicates 8-oxo-2'-deoxyguanosine immunoreactivity; blue indicates nuclear counterstain. (B) Quantitative real-time RT-PCR analysis of *Sod1*, *Cat*, and *Gpx1* mRNA extracted from 8 to 10-week old *Sod2^{L/L}* (L/L) or *Sod2^{-/-}* (-/-) lactating mammary glands. Results normalised first to 18s mRNA then to *Sod2^{L/L}* *Sod1* mRNA by the ddCT method.

Figure 3.

The loss of *Sod2* has no apparent effect on virgin mammary development. (A) Low magnification (4×) of hematoxylin stained 10-week old *Sod2^{L/L}* and *Sod2^{-/-}* whole mounted virgin mammary glands. (B) High magnification (40×) of hematoxylin and eosin (H&E) stained 10-week old *Sod2^{L/L}* and *Sod2^{-/-}* virgin mammary glands. Images reviewed by pathologist demonstrate no significant changes.

Figure 4.

Reproductive stages of mammary gland development including proliferation, lobuloalveolar differentiation, and lactation are apparently unaffected by *Sod2* loss. (A) High magnification (40×) of hematoxylin and eosin (H&E) stained 7-day postpartum *Sod2^{L/L}* and *Sod2^{-/-}* lactating mammary glands. (B) High magnification (40×) of hematoxylin and eosin (H&E) stained involuted *Sod2^{L/L}* and *Sod2^{-/-}* mammary glands. Images reviewed by pathologist demonstrate no significant changes.

Figure 5.

Conditional mammary *Sod2* knock-out mothers bear normal numbers of healthy pups and sustain multiple litters. (A) Average counts of number of pups per litter throughout reproductive lifetime in *Sod2*^{+/L} and *Sod2*^{-/-} mice. Black bar = *Sod2*^{+/L}, Grey bar = *Sod2*^{-/-}. (B) Average weight per pup at varying time points until weaning. Black bar = *Sod2*^{+/L}, Grey bar = *Sod2*^{-/-}. Six mice per experiment were analysed; data are shown as mean and s.d.

Table I

Sod2 knock-out remains penetrant throughout multiple litters.

Litter	<i>Sod2</i> ^{L/L}	<i>Sod2</i> ^{-/-}
1	1.01 ± 0.14	0.08 ± 0.01*
2	0.98 ± 0.15	0.05 ± 0.02*
3	0.95 ± 0.11	0.09 ± 0.02*
4	1.03 ± 0.06	0.06 ± 0.03*

Quantitative real-time RT-PCR analysis of *Sod2* mRNA extracted from *Sod2*^{L/L} or *Sod2*^{-/-} lactating mammary glands after respective litter number. Results normalised first to 18s mRNA then to respective *Sod2*^{L/L} by the ddCT method. Three mice per experiment were analysed; data are shown as mean and s.d. Where applicable, * = $p < 0.01$ by Student's *t*-test versus *Sod2*^{L/L}.