**Timing and duration of nursing from birth affect
neonatal porcine uterine matrix metalloproteinase 9 and
tissue inhibitor of metalloproteinase 1**

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Abstract

Nursing for 2 d from birth supports neonatal porcine uterine and cervical development. However, it is not clear how timing or duration of lactocrine signaling from birth (postnatal day = PND 0) affect development of neonatal female reproductive tract (FRT) tissues. Therefore, studies were conducted to determine effects of age at first nursing and duration of nursing from birth on specific elements of the matrix metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (TIMP) system in uterine and cervical tissues at PND 2. When nursing was initiated at 0 h or 30 min of age, targeted proteins, including proMMP9 and MMP9, were detected in uterine and cervical tissues on PND 2, as was uterine TIMP1. However, these proteins were undetectable when nursing was delayed for 12 h, and when gilts were fed milk replacer for 48 h from birth. Increasing the duration of nursing from 30 min to 12 h from birth increased uterine ($P < 0.05$) and cervical ($P < 0.001$) MMP9 levels to those observed in gilts nursed for 48 h. Similarly, uterine TIMP1 levels increased with duration of nursing. Uterine MMP2 levels were detectable but unaffected by age at first nursing or duration of nursing from birth. Uterine MMP2 and MMP9 activities, monitored by zymography, reflected immunoblotting data. Results provide evidence for the utility of MMP9 and TIMP1 as markers of age- and lactocrine-sensitive porcine female reproductive tract development.

Keywords: pig, uterus, neonatal, lactocrine, matrix metalloproteinases

40 1. Introduction

41 Nursing supports neonatal porcine reproductive tract development by delivery of milk-
42 borne bioactive factors from mother to offspring as proposed in the lactocrine
43 hypothesis [1,2]. Imposition of the lactocrine-null condition for two days from birth
44 (postnatal day = PND 0), by feeding gilts a commercial milk replacer instead of
45 colostrum, altered patterns of cervical [3] and uterine [4,5] gene expression at
46 transcriptional and/or translational levels on PND 2. These effects persisted to PND 14
47 even after gilts were returned to nursing on PND 2 [4]. In the absence of lactocrine
48 signaling for two days from birth, endometrial growth and uterine gland development
49 were markedly retarded by PND 14 [4]. Thus, porcine uterine and cervical development
50 are supported by lactocrine signals during the first two days of neonatal life. Whether a
51 critical window for lactocrine signaling supportive of female reproductive tract
52 development exists within this 48 h neonatal period remains to be determined.

53 Colostrum is rich in milk-borne bioactive factors and the composition of porcine
54 colostrum, like that of other mammals [6], changes throughout the course of lactation
55 [7,8]. Further, the composition of colostrum and milk can vary from anterior to posterior
56 mammary glands [9], and consumption of colostrum by newborn pigs can be delayed if
57 sows fail to initiate lactation or to produce sufficient colostrum for the litter [10,11]. In
58 pigs, a window for transmission of colostral milk-borne bioactive factors to offspring is
59 open before gut closure and is estimated to occur within the first 48 h after birth [12].
60 Data indicating that lifetime fecundity of adult female pigs is markedly reduced when
61 their colostrum consumption is minimal on the first day of neonatal life [7] emphasizes

the importance of these issues for reproductive development. Whether timing of colostrum consumption relative to birth, or duration of nursing from birth influences lactocrine signaling in the neonatal uterus is unknown.

A recent analysis of the neonatal porcine uterine transcriptome on PND 2 showed that expression of multiple matrix metalloproteinase (MMP) and associated genes was affected by age and lactocrine signaling between birth and PND 2 [5]. Matrix metalloproteases are proteolytic enzymes that facilitate extracellular matrix (ECM) remodeling and tissue growth during development and disease [13]. The activity of MMPs is regulated tightly by coexpression of specific tissue inhibitors of metalloproteinases (TIMPs) [14]. Uterine [15] and cervical [3] MMP9 protein levels increased in nursed gilts as compared to gilts fed milk replacer for two days from birth. Whether the MMP/TIMP system is affected by the timing or duration of nursing from birth in developing porcine female reproductive tract tissues is unknown. Objectives of this study were to determine the effects of: (1) age at first nursing; and (2) duration of nursing from birth on specific elements of the cervical and uterine MMP/TIMP system at PND 2.

2. Materials and methods

2.1. Animals

Gilts (*Sus scrofa domesticus*) born from an established herd of crossbred (Duroc, Hampshire, Yorkshire, and Landrace) sows were raised at the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University. All procedures involving animals were reviewed and approved by the Rutgers Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of

Agricultural Animals in Research and Teaching (2010; Federation of Animal Science Societies, Champaign, IL, USA). Gilts were weighed at birth and included in the experiment if body weight was 1.3 kg or greater, based on studies suggesting that lower weight pigs display below average postnatal development [16]. Body weights were recorded daily until PND 2. Gilts that nursed remained with their dams, whereas those fed milk replacer (Advanced Liqui-Wean®; Carpentersville, IL, USA) were housed in a separate pen in the same environment and in close proximity to their dams and littermates. All pens were equipped with heat lamps. Care was taken to insure that treatments were balanced for potential effects of litter (n=17), and that sows were nursing litters of similar size.

2.2. Experimental design and tissue collection

Two experiments were conducted. To determine effects of age at first nursing on uterine levels of targeted proteins (MMP2, MMP9 and TIMP1) at PND 2, crossbred gilts were assigned randomly at birth to treatment groups (n= 5/group) in which gilts were either: (1) nursed *ad libitum* for 48 h; (2) gavage-fed milk replacer from birth and switched to nursing at 0.5 h of age; (3) gavage-fed milk replacer from birth and switched to nursing at 12 h of age; or (4) gavage-fed milk replacer (30 ml/kg BW/2h) for 48 h from birth (Fig. 1A). To determine effects of the duration of nursing from birth on the same endpoints, gilts (n= 5-6/group) were assigned randomly at birth to be either: (1) gavage-fed milk replacer for 48 h; (2) nursed *ad libitum* for 0.5 h from birth; (3) nursed for 12 h from birth and then gavage-fed milk replacer through 48 h; or (4) nursed *ad libitum* for 48 h from birth (Fig. 2A). In all experiments, pigs were euthanized at 48 h after birth

when uterine and cervical tissues were removed, trimmed free of associated tissues, immersed in RNALater and stored at -80°C.

2.3. Western blot analyses

Whole cervical and uterine tissues (20 mg) were homogenized in lysis buffer (1% Triton X-100, 10% glycerol, 150 mM Tris-HCl, 300 mM NaCl, and 1 mM MgCl₂; pH 7.5) and incubated at 4°C for 30 min. Individual homogenates representing each tissue sample were centrifuged (12000g at 4°C for 10min), and protein supernatants were stored at -20°C. Total protein concentration was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Tissue proteins (20 µg) were resolved on 12% (total monomer) NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA) under reducing conditions followed by transfer onto nitrocellulose membranes (0.45-µm pore size; Bio-Rad Laboratories). Individual samples from each animal for each treatment were run in duplicate. Treatment effects on tissue MMP9 (pro 92 kDa, active 84 kDa), MMP2 (pro 72 kDa, active 66 kDa), and TIMP1 (26 kDa) protein levels were evaluated after immunoblotting using actin (43 kDa) as a loading control.

Nitrocellulose membranes were blocked with 10% nonfat milk powder (NFMP) in TBST buffer [25 mM Tris (pH 7.5), 0.14 M NaCl, 3 mM KCl, and 0.05% Tween 20] for 1 h at room temperature. Membranes were then incubated with either mouse antihuman MMP2 (1:1000; IM33; EMD Millipore, Billerica, MA), mouse antihuman MMP-9 (1:100; Ab-1; EMD Millipore), rabbit antihuman TIMP1 (1:1000; C20; Santa Cruz Biotechnology, Dallas, TX), or goat antihuman actin antibody (1:3000, C11; Santa Cruz Biotechnology) in TBST-5% NFMP overnight at 4°C. Human MMP family proteins are well-conserved across species and homology between human and pig proteins is high

(96% for MMP2, 80% for MMP9 and 84% for TIMP1; BLAST, US National Center for Biotechnology Information). Antibodies directed against human MMP2, MMP9 and TIMP1 were validated for use in the pig uterus [17,18].

Membranes were washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated goat antimouse secondary antibody (1:2000 for MMP2 and MMP9; Invitrogen, Grand Island, NY), Trueblot HRP mouse antirabbit IgG (1:2000 for TIMP1; Rockland, Limerick, PA) or rabbit antigoat IgG (1:2000 for actin; Invitrogen) in TBST-5% NFMP for 1 h at room temperature. After washing in TBST, membrane-bound antibodies were detected by enhanced chemiluminescence (Renaissance Western Blot Chemiluminescence Reagent Plus; PerkinElmer Life Sciences, Waltham, MA), per manufacturer recommendations. Antibody specificity was confirmed by omission of primary antibodies in immunoblot analyses. Incubation with secondary antibody alone showed no cross reactivity with cervical or uterine proteins. Both MMP2 and MMP9 are expressed in the neonatal [15] and prepubertal pig uterus [17] and increase during pregnancy [19,20]. Thus, positive controls used for MMP protein studies included the PND 2 uterus for MMP2, and the pregnant sow uterus (gestation d 90) for MMP9 [21]. Bovine recombinant TIMP1 (EMD Millipore, Billerica, MA) was used as a TIMP1 positive control on Western blots. Negative controls included substitution of an irrelevant mouse IgG (for MMP2 and MMP9) or rabbit IgG (for TIMP1) for primary antibodies in each case as appropriate. Additionally, blots were incubated with secondary antibody alone to evaluate potential secondary antibody cross reactivity with uterine or cervical proteins at expected molecular sizes for targeted proteins.

Chemiluminescent signals were quantified densitometrically from film using Scion Image for Windows (Scion Corp., Frederick, MD). X-Omatic films were purchased from American Imaging (South Plainfield, NJ, USA). All data were corrected for background signals and expressed as a target protein-to-actin ratio. Densitometric analyses of total MMP2 (pro 72 kDa, active 66 kDa) and total MMP9 (pro 92 kDa, active 84 kDa) protein and activity are reported. When immunoblot signals were below background under identical exposure conditions, data were recorded as not detectable (ND).

2.4. Gelatin zymography

Zymography was performed as described previously [17] with modifications. Briefly, uterine proteins (30 µg) were mixed with equal amounts of SDS sample buffer and loaded under nondenaturing conditions using precast polyacrylamide zymogram gels supplemented with 1% gelatin as the proteinase substrate (Novex® 10% Zymogram Gelatin Protein Gels, Invitrogen). After electrophoresis, gels were washed in renaturing buffer (2.7% (w/v) Triton X-100) for 30 min to remove SDS, and incubated in developing buffer [50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM CaCl₂, 0.02% (w/v) Brij 35] overnight at 37°C. The positive control for MMP2 activity was PND 2 uterine tissue, while a lysate from MCF-7 cells treated with 12-O-Tetradecanoylphorbol-13-acetate was used to confirm MMP9 activity [22]. Gels were stained with 0.1% Coomassie brilliant blue R 250 followed by destaining in order to obtain a clear lytic band against a blue background, indicating enzymatic activity. Using this method, latent and active MMP2 and MMP9 were visualized based on their respective molecular sizes [23]. Relative gelatinolytic activity was quantified by densitometry and expressed as the optical density of the inverted lytic zone measured by Scion image.

2.5. Statistics

Data were subjected to analyses of variance using General Linear Models Procedures (SAS 2013, Cary, NC) for a mixed model as appropriate. Preplanned comparisons were performed to determine main effects of age at first nursing and duration of nursing. Comparisons for age at first nursing included: (1) nursing at 0 h vs no nursing (replacer feeding for 48 h); (2) nursing begun at 0 h vs nursing begun at 0.5 h; and (3) nursing begun at 0 h vs nursing begun at 12 h on responses assessed at PND 2. Comparisons for duration of nursing studies were: (1) 48 h of nursing vs no nursing (replacer feeding for 48 h); (2) 48 h of nursing vs nursing for 0.5 h from birth; and (3) 48 h of nursing vs nursing for 12 h from birth on responses assessed at PND 2. Error terms were identified based upon expectations of the mean squares for error. Data are presented as least squares means $\pm$ SEM.

3. Results

3.1. Effects of age at first nursing on neonatal uterine MMP9, MMP2, and TIMP1 protein levels

Results of the study conducted to determine effects of age at first nursing on uterine MMP2, MMP9 and TIMP1 protein levels on PND 2 are presented (Fig. 1B-G). Immunoblotting revealed that proMMP2 (72 kDa) was detected in uteri from all nursed and replacer-fed gilts (Fig. 1B). A weaker, 66 kDa protein band, corresponding in size to active MMP2, was evident in all treatment groups, and was more pronounced when nursing began at 0 h and 0.5 h (Fig. 1B). However, overall densitometric analyses indicated that total uterine MMP2 (72 kDa + 66 kDa) was not affected by age at first nursing (Fig. 1E). **Immunoreactive bands detected for each of the targeted proteins in**

positive control samples (Fig. 1B-D) were not detected when irrelevant mouse IgG (MMP2 and MMP9) or rabbit IgG (TIMP1) was substituted for the primary antibody (negative control), confirming primary antibody specificity. Absence of an actin signal in negative and positive control lanes, illustrated specifically in Fig. 1D, reflects the fact that a purified TIMP1 protein preparation was used here and did not contain actin. In blots incubated with secondary antibody alone there was no evidence of cross reactivity with uterine or cervical proteins at expected molecular sizes for targeted proteins (data not shown).

Distinct proMMP9 (92 kDa) and active MMP9 (84 kDa) proteins were detected in uterine tissues obtained from gilts on PND 2 at equivalent levels regardless of whether nursing began at 0 h or 0.5 h (Fig. 1C, F). However, when nursing was delayed to 12 h neither latent nor active MMP9 proteins were detected (Fig. 1C, F). These results were similar to those observed for gilts fed replacer for 48 h from birth (no nursing).

Given results for MMP9, treatment effects on uterine TIMP1, the primary regulator of MMP9 [24], were evaluated. Consistently, treatment effects on uterine TIMP1 (28 kDa, Fig. 1D) levels were similar to those observed for total uterine MMP9. Uterine TIMP1 protein was detectable when nursing began at 0 h or 0.5 h, but not in tissues obtained on PND 2 when nursing was delayed until 12 h after birth, or in 48 h replacer-fed gilts (no nursing; Fig. 1D, G).

3.2. Effects of nursing duration on neonatal uterine MMP9, MMP2 and TIMP1 protein levels

Results of the study conducted to determine effects of the duration of nursing from birth on targeted uterine protein levels at PND 2 are presented (Fig. 2B-G). A prominent immunoreactive proMMP2 band at 72 kDa was detected in uteri from all nursed and replacer-fed gilts (Fig 2B). A less intense 66 kDa signal, corresponding to active MMP2 protein, was observed for the positive control. This 66 kDa immunoreactive MMP2 band was difficult to distinguish from the more prominent 72 kDa proMMP2 band in porcine uterine protein extracts. Overall, total uterine MMP2 protein levels were unaffected by nursing duration (Fig. 2E). Uterine MMP9 proteins were barely detectable at PND 2 in gilts that did not nurse or that nursed for only 0.5 h from birth. However, when duration of nursing was extended to 12 h, intense immunoreactive bands corresponding to proMMP9 (92 kDa) and MMP9 (84 kDa) were detected consistently (Fig. 2C). Total uterine MMP9 levels at PND 2 in gilts nursed for 12 h from birth were similar to those observed for gilts nursed for 48 h (Fig. 2C, F). Increasing the duration of nursing from birth to 12 h increased ($P < 0.05$) total MMP9 protein to levels similar to those observed in gilts nursed for 48 h (Fig. 2F). Duration of nursing effects on uterine TIMP1 levels (Fig. 2D) mirrored data for MMP9 in that TIMP1 was only detected in gilts that nursed for 12 h or 48 h from birth (Fig. 2D, G).

3.3. Effects of age at first nursing and nursing duration on neonatal uterine MMP2 and MMP9 activity

Results of gelatin zymography analyses for latent and active uterine MMP2 and MMP9 are presented in Figure 3. For age at first nursing, gelatinolytic bands corresponding to proMMP2 (72 kDa) and MMP2 (66 kDa) were detected in uteri from gilts in all

experimental groups (Fig. 3A) and total MMP2 activity was similar at all time points studied (Fig. 3C). In contrast, zymography revealed both uterine proMMP9 and MMP9 activity at PND 2 in gilts that began nursing at 0 h or 0.5 h of age (Fig. 3B & D). However, when age at first nursing was delayed to 12 h after birth, neither latent nor active MMP9 activities were detected. These effects were comparable to those observed for gilts fed replacer for 48 h from birth (no nursing; Fig. 3D).

For duration of nursing, there was no effect of nursing or replacer feeding on uterine proMMP2 or MMP2 activity (Fig. 3E & G). However, uterine proMMP9 (92 kDa) and MMP9 (84 kDa) activities were low in gilts fed replacer for 48 h (no nursing) or that nursed for only 0.5 h from birth (Fig. 3F). Increasing the duration of nursing from birth to 12 h increased ($P < 0.001$) total MMP9 activity to levels similar to those observed for gilts nursed for 48 h (Fig. 3H).

3.4. Effects of age at first nursing and duration of nursing on neonatal cervical MMP9 protein levels

Results of studies designed to determine effects of age at first nursing and duration of nursing on cervical MMP9 protein levels are presented in Figure 4. Data obtained for cervical tissues were complementary to those obtained for uterine tissues. With respect to effects of age at first nursing, both proMMP9 (92 kDa) and MMP9 (84 kDa) proteins were detected in cervical tissues obtained from gilts on PND 2 at equivalent levels, regardless of whether nursing began at 0 h or 0.5 h (Fig. 4A & C). However, when nursing was delayed to 12 h, neither latent nor active MMP9 proteins were detected in

the cervix. These results were similar to those observed for gilts fed replacer for 48 h from birth.

For effects of the duration of nursing from birth, immunoreactive bands corresponding to latent and active cervical MMP9 proteins were detectable on PND 2 in gilts that had not nursed, and increased ($P < 0.05$) after only 0.5 h of nursing (Fig. 4B & D). Extending the duration of nursing to 12 h from birth further increased ($P < 0.001$) total MMP9 levels, which were similar to those observed for gilts nursed for 48 h (Fig. 4D).

4. Discussion

Uterine development in the neonatal pig requires interactions between stroma and epithelium that define the endometrial morphogenetic program [25,26]. Stromal-epithelial interactions that support development of specialized structures such as uterine glands involve cell-cell and cell-ECM interactions. Between birth and PND 3, the neonatal porcine uterine wall is transitioning to a morphogenetically active state [26, 27], as luminal endometrial epithelium differentiates into glandular epithelium, and nascent glandular epithelial buds proceed through tubulogenesis and beyond to form endometrial glands, required to support pregnancy in adults [27]. Matrix metalloproteinases and their tissue inhibitors regulate synthesis and degradation of the ECM. Through such effects the MMP/TIMP system supports morphogenetic events including tubulogenesis and branching morphogenesis in several tissues [28], as well as remodeling of the adult uterine wall during the ovarian cycle, implantation, and pregnancy [29].

289 In the developing neonatal mouse uterine wall, several MMPs and TIMPs were
290 localized to endometrial epithelial and/or stromal cells [30]. Consistently, a recent
291 analysis of the porcine uterine transcriptome between birth and PND 2 revealed that
292 elements of the plasminogen-activating network, including MMPs and TIMPs, were both
293 age- and lactocrine-sensitive [5]. Results presented here extend these studies by
294 showing that both age at first nursing and duration of nursing are important for neonatal
295 uterine MMP9/TIMP1 expression. This is consistent with a role for MMPs and TIMPs in
296 remodeling the neonatal uterine wall [30].

297 Imposition of the lactocrine-null state for 48 h from birth altered patterns of cervical [3]
298 and uterine [5,15] gene expression by PND 2 and inhibited endometrial adenogenesis
299 by PND 14 in the pig [4]. Further, minimal colostrum consumption by newborn gilts is
300 associated with reduced lifetime fecundity [7]. Collectively, these observations reinforce
301 the idea that the first 48 h of neonatal life is an important time for lactocrine signaling in
302 support of porcine uterine and cervical development. Taken together with present data
303 for uterine MMP9/TIMP1 and cervical MMP9, results suggest that the lactocrine-
304 sensitive porcine uterine developmental program is defined by PND 2, and that
305 colostrum consumption within 12 h of birth ensures that this program is established
306 correctly. Present results also indicate that both age at first nursing and duration of
307 nursing from birth are important to ensure that a normal uterine developmental program
308 evolves within 48h of birth.

309 Neither age at first nursing nor duration of nursing affected MMP2 responses in
310 uterine or cervical tissues. Results are consistent with studies indicating that MMP2 is
311 expressed constitutively in other systems, including fetal membranes and placenta

[19,31]. The fact that MMP2 protein levels were not age- or lactocrine-sensitive is consistent with previous observations for MMP2 in the uterus [15] and cervix [3]. As expected, uterine MMP9 activity, monitored by gelatin zymography, reflected results for immunoblotting data for age at first nursing in that both MMP9 protein and activity were barely detectable when nursing was delayed to 12 h after birth, and in gilts fed replacer for 48 h (no nursing). Likewise, for duration of nursing, expression of MMP9 protein mirrored MMP9 activity. Both were low to undetectable in gilts that did not nurse or nursed for only 0.5 h from birth.

Timing and duration of nursing from birth affect both the quality and amount of colostrum consumed by neonates and, therefore, the relative abundance and nature of lactocrine inputs available to affect postnatal developmental events. When age at first nursing was delayed to 4 h after birth in neonatal pigs, circulating immunoglobulin levels were decreased by half when compared to littermates that began nursing at birth [32]. This is consistent with the report that colostral protein concentration declines linearly during the first 12 h after parturition [8]. Thus, a delay in colostrum consumption from birth will reduce the amount of protein and, by association, lactocrine-active MbFs available to neonates.

The impact of nursing from birth, as well as the quality and/or composition of colostrum/milk, on patterns of somatic development is documented for several species. In the rat, differentiation of pituitary prolactin-secreting cells requires a milk-borne, maternal (lactocrine) signal delivered into the neonatal circulation within the first few days of lactation [33,34]. When newborn rats were deprived of early milk from birth by cross fostering to dams that had given birth a week earlier, mammatrope differentiation

was impaired and the secretory function of prolactin-releasing cells was altered in adulthood [35]. More recently, data for the mouse [36] showed that altering levels of specific milk chemokines affects patterns of hippocampal cell proliferation in nursed offspring, with long-term effects on adult spatial memory. In neonatal calves, delaying colostrum intake by one day altered plasma concentrations of metabolic hormones including gastrin, insulin and IGF-1 [37], and changed plasma fatty acid and fat-soluble vitamin status [38]. Data for the tammar wallaby [39] indicate that timing of milk consumption, as well as milk composition and rate of milk production determine patterns of pouch young growth and dictate offspring phenotype. These observations reinforce the importance of understanding how timing and duration of nursing affect mammalian developmental programming.

Until now, the duration of nursing from birth required to establish the normal developmental program governing organization of porcine female reproductive tract tissues was unexplored. However, nursing for as little as 1 h from birth was sufficient to support establishment of passive immunity in newborn pigs [32]. Consistently, data for the porcine cervix [40] indicate that a single feeding of 0 h colostrum at birth affects patterns of cell proliferation positively by 12 h postnatal. These observations support the idea that lactocrine transmission of milk-borne bioactive factors and associated effects of lactocrine-active factors on female reproductive tract tissues occur within 12h of birth. Present data provide evidence for the utility of MMP9 and TIMP1 as markers of age-and lactocrine-sensitive porcine female reproductive tract development. Results show that increasing the duration of nursing from 0.5 h to 12 h from birth supported expression of uterine MMP9 and TIMP1, as well as cervical MMP9 at levels equivalent

to those observed in gilts nursed for 48 h from birth. Thus, nursing for 12 h from birth is sufficient to provide lactocrine support for establishment of normal MMP9 and TIMP1 expression in the developing neonatal porcine uterus.

Acknowledgements

This research was supported by USDA-NIFA NRI Grants 2007-35203-18098 and 2013-67016-20523 to FFB and CAB and NSF-EPS-1158862 to FFB. CAB and FFB contributed equally to this work.

The authors thank the Rutgers Animal Care Program and the Rutgers undergraduate Sow Watch Club for their contributions to these studies.

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Figure Legends

Figure 1. Effects of age at first nursing on porcine uterine MMP2, MMP9 and TIMP1 protein at PND 2. **(A)** Experimental design; n=5 animals per group. Representative immunoblots for **(B)** proMMP2 (72 kDa) and MMP2 (66 kDa); **(C)** proMMP9 (92 kDa) and MMP9 (84 kDa); and **(D)** TIMP1 (28 kDa) are shown with their respective negative (-) and positive (+) controls as described in Materials and methods. Absence of an actin signal in (-) and (+) control lanes (Fig. 1D) reflects the fact that a purified TIMP1 protein preparation was used here and did not contain actin. Densitometric data for the relative expression of total uterine **(E)** MMP2 (72 + 66 kDa); **(F)** MMP9 (92 + 84 kDa) and **(G)** TIMP1 in relation to actin are presented as $LSM \pm S.E.M.$ Signals below the detection range are marked as non-detectable (ND). Bars with different letters denote differences at $P < 0.05$.

Figure 2. Effects of duration of nursing on porcine MMP2, MMP9 and TIMP1 protein at PND 2. **(A)** Experimental design; n=5 animals per group. Representative immunoblots for **(B)** proMMP2 (72 kDa) and MMP2 (66 kDa); **(C)** proMMP9 (92 kDa) and MMP9 (84 kDa); and **(D)** TIMP1 (28 kDa) are shown with their respective positive (+) controls as described in Materials and methods. Densitometric data for the relative expression of total uterine **(E)** MMP2 (72 + 66 kDa); **(F)** MMP9 (92 + 84 kDa) and **(G)** TIMP1 in relation to actin are presented as $LSM \pm S.E.M.$ Signals below the detection range are marked as non-detectable (ND). Bars with different letters denote differences at $P < 0.05$.

Figure 3. Effects of age at first nursing and duration of nursing on porcine uterine MMP2 and MMP9 activity at PND 2. Representative zymograms illustrating the effects of age at first nursing for **(A)** proMMP2 (72 kDa) and active MMP2 (66 kDa) and **(B)** proMMP9 (92 kDa) and MMP9 (84 kDa) are shown with their respective positive (+) controls as described in the Materials and methods. Densitometric data for the relative expression of **(C)** total MMP2 (72 + 66 kDa) and **(D)** total MMP9 (92 + 84 kDa) are presented as $\text{LSM} \pm \text{S.E.M.}$ Representative zymograms showing the effects of duration of nursing for **(E)** proMMP2 (72 kDa) and MMP2 (66 kDa) and **(F)** proMMP9 (92 kDa) and MMP9 (84 kDa) are shown. Densitometric data for the relative expression of **(G)** total MMP2 (72 + 66 kDa) and **(H)** total MMP9 (92 + 84 kDa) are presented as $\text{LSM} \pm \text{S.E.M.}$ Signals below the detection range are marked as non-detectable (ND). Bars with different letters denote differences at $P < 0.001$.

Figure 4. Effects of age at first nursing and duration of nursing on porcine cervical MMP9 protein at PND 2. Representative immunoblots showing the effects of **(A)** age at first nursing and **(B)** duration of nursing on cervical proMMP9 (92 kDa) and MMP9 (84 kDa) are shown. For **(C)** age at first nursing and **(D)** duration of nursing densitometric data for the relative expression of total cervical MMP9 (92 + 84 kDa) are presented as $\text{LSM} \pm \text{S.E.M.}$ All signals below the detection range are marked as non-detectable (ND). Bars with different letters denote differences (a vs b, $P < 0.05$; b vs c, $P < 0.001$).

Fig 1

Fig 2

Fig 3

Fig 4