The potential of identifying replacement gilts by screening for lipid biomarkers in reproductive tract swabs taken at weaning

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ABSTRACT

Sow longevity affects economic returns to pork producers. The cost of gilt replacements is substantial and sows with greater than three litters have lower costs per pig produced. An early marker of reproductive potential would facilitate early identification of superior females, and likely increase sow longevity. Gilts raised in small litters have greater reproductive competence, but mechanisms associated with increased reproductive responses are not fully understood. Here, early postnatal development of the gilt’s reproductive tract is described, and a brief review of literature is presented to support that factors in colostrum regulate the developmental trajectory of the gilt’s uterine tissues. We propose that, similar to the uterus, nutritional environment likely affects the postnatal developmental programme of the vagina. A metabolomics approach, multiple reaction monitoring -profiling, for biomarker discovery is described, along with evidence that lipids present in vaginal samples are differentially expressed in gilts exposed to colostrum versus milk replacer fed. These exploratory studies indicate that the vaginal cell lipidome may reflect the postnatal nutritional environment, which defines to a large extent the gilt’s reproductive potential. Together findings support further investigations to identify biomarkers predictive of fertility outcomes in the metabolome of gilt reproductive tracts.

Introduction

A breeding herd’s economic efficiency has a direct relationship to sow removal or culling rates. According to PIGChamp’s 2016 year-end report, the average culling rate for sows and gilts was staggeringly high at 44%, with reproductive failure indicated as the leading cause of culling (NAHMS, 2012). Simulation studies of pork production systems found the farms with the lowest replacement rates are the most profitable (Stalder et al. 2004). Economic analysis of production costs found that a gilt must remain in the breeding herd for three parities to reduce the substantial replacement costs (Stalder et al. 2004). Therefore, high culling rates are a concern to modern commercial herds because a smaller percentage of sows are producing in the most profitable parities.

The high culling rates translate to production systems producing large numbers of gilts to replace the culled sows on farms. While it is common for pork producers to select replacement gilts based on their phenotype (body type, feet and leg structure, number and placement of teats), less emphasis has been placed on reproductive selection (Lowe 2010), despite this being the main reason for culling of mature sows. Reproductive selection is the process of identifying the gilts that will have the highest reproductive potential for lifetime performance. Farms that do use reproductive potential to select replacement sows primarily rely on identifying gilts that come into estrus soon after boar exposure begins. Gilts that come into heat within 30 days of boar exposure have more piglets in their first litter and are more likely to stay in the herd (Lowe 2010). However, at this point, producers have already invested six months into gilts that fail to come into heat and never produce a single offspring. Lack of an ability to identify replacement gilts early in development results in not only culling prior to their first mating, but also after only one or two parities, which is prior to profitability. Therefore, an early marker of reproductive potential would facilitate early identification of superior females, increase reproductive performance and reduce culling in the sow herd.

Early postnatal environment and its relation to sow longevity

Although sow longevity is a trait with substantial economic value, genetic improvement for sow longevity has challenges (Stalder et al. 2003; Engblom et al. 2015). The longevity of a sow in the herd is dependent on a multitude of factors (Stalder et al. 2004), some of which begin at or before birth. Industry wide, there has been an increase in the number of piglets born per litter through genetic selection which has resulted in an increased variation in birthweights as well as variation in long-term robustness of pigs (Foxcroft et al. 2007; Knauer and Hostetler 2013; Rutherford et al. 2013). Heavier pigs at birth grow faster and have higher preweaning survival rates than their lighter counterparts (Lawlor et al. 2002; Schinckel et al. 2009). In addition, gilts raised in large litters...
produce fewer piglets as breeding sows than those raised in small litters (Robison 1981; Van der Steen 1985). To understand the potential mechanisms determining this fate, investigators paired litters at birth and reduced one litter by cross-fostering to the other to result in paired-sows suckling litters of 6 or 14 piglets. Gilts reared in the larger litters had 1.2 less piglets born alive per litter than those reared in litters of six. Similarly, replacement gilts reared in litters of 7 versus 11 piglets had increased farrowing rates (88.7% vs. 83.3%), increased number born alive (11.0 vs. 10.5) and increased longevity in the herd (35.5% vs. 17.3% to parity 6). Replacement gilts reared in the smaller litters were also heavier at weaning, and maintained their weight advantage at the time of entering the sow herd. Additionally, there was a greater percentage of sows that farrowed a second litter when raised in smaller litters as gilts (Flowers 2008). Although traits improved by rearing in smaller litters (farrowing rates, sow longevity and litter size) are economically important to pork producers, the practicality of strategic cross-fostering to create litter sizes of 7 piglets when each sow is producing an average of 14 pigs per litter is challenging and unrealistic.

Postnatal reproductive tract development and the impact of colostrum

In pigs, reproductive tract development is incomplete at birth, and a significant amount of reproductive tract development occurs in the early postnatal period in gilts (Bartol et al. 2006). The morphological and molecular changes that occur in the porcine uterus and cervix in the early postnatal period have been extensively described (Bartol et al. 1993; Spencer, Bartol, et al. 1993; Spencer, Wiley, et al. 1993; Tarleton et al. 1998, 1999, 2001; Bartol et al. 2006; Miller et al. 2013; Camp et al. 2014; Rahman et al. 2016), and here we briefly summarize this work.

At birth, the uterine wall consists of a simple luminal epithelium overlying a loosely organized stroma and developing myometrium in pigs (Bartol et al. 1993; Spencer, Bartol, et al. 1993; Spencer, Wiley, et al. 1993; Tarleton et al. 1999). Endometrial glands begin to develop during the first weeks of neonatal life and are characterized by two periods of development: (1) the infantile period, between birth and postnatal day 7, which encompasses the onset of endometrial gland development; and (2) the proliferative period, between postnatal days 7 and 14, when DNA synthesis in new glandular epithelium is maximal. Along with remodelling events resulting in formation of endometrial glands, there are changes in patterns of uterine protein synthesis (Spencer, Bartol, et al. 1993; Bartol F. F. et al. 2006) and onset of endometrial oestrogen receptor-α (ER) expression (Bartol et al. 1993; Spencer, Bartol, et al. 1993; Bartol et al. 2006). Spatial relationships between ER-positive and ER-negative stromal and epithelial cells change between birth and postnatal day 56 (Tarleton et al. 1999, 2001). The endometrium is ER negative at birth, by postnatal day 15 the stroma and glandular epithelium, but not luminal epithelium, express ER. After postnatal day 30, a mature endometrial ER architecture is evident, and is characterized by all major cell types, including stroma, glandular and luminal epithelium being ER-positive (Bartol et al. 1993; Tarleton et al. 1998).

Colostrum not only meets nutritional needs of neonates, but also contains bioactive components that stimulate development and immune function, that is, initiate lactocrine signalling. The amount of colostrum consumed by neonatal gilt must be adequate for the lactocrine signalling and developmental programming needed for them to achieve their genetic potential for reproductive performance (Bartol et al. 2013; Flowers 2015), with multiple studies demonstrating that exposure to colostrum sets the developmental trajectory of the gilts’ reproductive tract. For example, profound histomorphological differences in uterine gland development (Flowers 2008; Frankshun et al. 2012; Bartol et al. 2013) as well as differences in endometrial gland and cervical cell gene expression (Bartol and Bagnell 2012; Frankshun et al. 2012; Bartol et al. 2013; Rahman et al. 2016) were found between gilts that were allowed to suckle the sow’s milk versus being fed milk replacer the first 48 h of life. Additional studies found that the hormone relaxin, which is produced by sows during farrowing, is transferred to neonates through colostrum. Intramuscular injection of relaxin every 6 h for 48 h had a significant impact on endometrial gland growth and increased expression of genes that stimulate uterine development (ESR1, VEGFA, BCL2) on postnatal day 14 in gilts that were suckled during the first 48 hours versus controls (suckled alone). In contrast, relaxin had no effect on uterine morphology or gene expression when piglets were fed milk replacer the first 48 h postnatal (Bagnell et al. 2008, 2009). Therefore, exposure to colostrum in the early post-partum is necessary to potentiate the effect of relaxin treatment on female reproductive tract development, and thus colostrum likely has multiple bioactive factors (yet to be identified) that profoundly affect the developmental trajectory of the suckling neonate.

The passive transfer of maternal immunoglobulins in colostrum can be estimated by an immunocrit assay, which measures immunoglobulins in neonate blood serum (Vallet et al. 2013). Piglets with low immunocrit values were found to have substantially lower survival rates, with immunocrit values accounting for four times the variation in preweaning survival than birth weight (Vallet et al. 2013). Immunocrit values were also associated with variables that affect sow productivity. Gilts with low immunocrit values required eight additional days to achieve puberty and had fewer piglets born alive over three parities than gilts with high immunocrit values (Vallet et al. 2015). Therefore, the reproductive performance of female piglets that do not receive sufficient colostrum, as indicated by low immunocrit value, is permanently impaired. This finding supports a causal link between impaired reproductive tract development, as seen in gilts fed milk replacer, and insufficient colostrum exposure in the early postnatal period.

Proposal to screen for biomarkers of fertility in vaginal swab samples

Economic analysis estimated that replacement gilts having limited colostrum intake cost the pork industry over $200 million each year (Engblom et al. 2015). The amount of colostrum produced by sows has not increased as a result of selection for increased litter size. Further, timing of exposure is
critical. Maximal absorption of colostrum components across neonatal intestine occurs within the first six hours after birth and decreases rapidly to completely disappear by 36 h (Lanza et al. 1995). This fact suggests that early postnatal vigour and competition for sows’ teats are factors that likely determine reproductive competence. Thus, identifying gilts in the large litters of the modern system that have consumed adequate amounts of colostrum is challenging because it is not completely explained by body weight or growth rates (Quiniou et al. 2002; Quesnel et al. 2012). Further, measuring immunocrit requires blood-sampling gilts at 24–48 h postnatal, which is not practical for large production systems, and immunocrit levels are currently a relatively subjective evaluation.

There is thus a need to identify non-invasive measures predictive of a gilt’s reproductive competence, and we have begun investigations to determine the efficacy of identifying lipid biomarkers of fertility in vaginal cell swabs taken at weaning. The vagina has the same developmental origin as the cervix and uterus and, similar to these organs, undergoes changes throughout a female’s lifetime (Cai 2009). The vagina is also similarly susceptible to developmental aberrations related to perinatal exposures (e.g. (Hoover et al. 2011)); and so its developmental trajectory is likely affected by early nutritional environment.

Use of vaginal cell swabs also has some precedence, as although components in blood are often used as biomarkers, there is growing interest as well as established protocols that use cells to non-invasively evaluate biomarkers of health and disease. For example, buffal cell samples have been evaluated to measure response to dietary lipids (Klingler et al. 2013), genotoxic exposure (Bolognesi et al. 2017), epigenetic changes associated with obesity (San-Cristobal et al. 2017) and early detection of Alzheimer’s Disease (Francois et al. 2014). Highly relevant to using vaginal smears is the use of cervical smears (i.e. Papanicolaou test, Pap smear) for screening for cytological and more recently molecular changes in cervical cells associated with human papilloma virus-HPV infection (Bergeron and von Knebel Doeberitz 2016; Spiryda et al. 2016). Plasma components have a tendency to vary by dietary intake or diurnal patterns, whereas cell membrane and cytoplasmic lipids account for more stable information obtained from metabolic transformations together with stabilized dietary contributions. Cellular lipids and metabolites can therefore be useful in examining metabolic influences (Ferreri et al. 2017).

**Metabolomics approach to biomarker discovery**

Biomarkers are objective, quantifiable characteristics of biological processes (Strimbu and Tavel 2010), and can be used to distinguish phenotypes (e.g. health or disease). Phenotype is determined by complex interactions between genotype, diet, nutrition, environmental exposure, lifestyle (in humans) – or management factors (in production animals) and gut microflora. The metabolome offers the most revealing real-time insights towards understanding phenotype as it captures important influences that go beyond the genome (Beger et al. 2016).

Several platforms are available for performing metabolomics, with liquid chromatography/mass spectrometry (LC/MS) most commonly used for discovery or untargeted analysis. Recent breakthrough untargeted metabolite profiling has enabled the quantification of tens of thousands of distinct metabolites in complex biological samples (e.g. blood and urine) (Beger et al. 2016). Although powerful, the volume of these data is currently overwhelming as only a small fraction of cellular metabolites currently have a defined or known function and are annotated in databases (Zamboni et al. 2015). Further, these current metabolomics approaches try to measure individual metabolites, which is slow and impractical. Multiple reaction monitoring (MRM)-profiling is a mass spectrometry (MS)-based metabolomics strategy recently developed for biomarker discovery by investigators at Purdue University (Ferreira et al. 2016; Cordeiro et al. 2017). MRM-profiling is based on screening chemical classes of small molecules instead of individual molecules. In particular, MRM-profiling uses the precursor and neutral loss scan modes of the mass spectrometer to run different experiments looking for fragmentation features (mass to charge ratios; m/z) related to specific chemical classes (Figure 1). In other words, MRM-profiling uses a chemical-based approach, which is to monitor molecules by their functional groups to discover, monitor and isolate prospective biomarkers. Monitoring functional groups rather than individual molecules has clear advantages, because although the function of every metabolite is currently not known, the functional groups of molecules have, for the most part, already been defined. For example, the membrane lipid phosphatidylycholine (PC) class has a choline head group. When lipids from this class are fragmented, a product ion characteristic of PC occurs at mass to charge ratio (m/z) of 184. Therefore, instead of recording thousands of product ion mass spectra and seeking molecules that include the ion of m/z 184, the precursor ion scan shows only molecules that actually have this group. This is in contrast to traditional metabolomics discovery methods, which are product ion scans performed across the entire mass range, and thus create very large datasets (Schwartz et al. 1990). The MRM-profiling data are much more manageable since individual metabolites occur in the many thousands, but functional group numbers are roughly 1000 times lower. Thus MRM-profiling gives ~103 less data and requires at least 10 times less instrument time than conventional methods since it is intended to cover just the informative part of the data space where metabolites may occur.

Another feature of MRM-profiling is that it is a two-step method. The first step is the discovery phase (Figure 1). During the discovery phase, a representative sample of each experimental group, such as a pooled sample of health or disease, is investigated to inform the researcher of functional groups present in the sample. In the second phase, the screening step of MRM-profiling, all the individual samples in both groups are investigated, but only for ion pairs detected in the discovery step. In other words, this makes MRM measurements extremely fast (no mass scanning) as samples are individually investigated for the ion pairs that the representative samples were shown to be characteristic of each class.

Similar to traditional MS-based metabolomics, MRM-generated profiles are queried for potential biomarkers that distinguish between groups using multivariate statistical methods (principal component analysis (PCA), cluster analysis).
as well as univariate methods (t-test or ANOVA, fold-change, Volcano plots). Data visualization is by heat maps and dendograms. Other methods recommended for biomarker discovery such as receiver operating characteristic (ROC) curves are also used. ROC curves are a common approach to selecting potential biomarkers (Xia et al. 2013). ROC curves play a central role in evaluating diagnostic ability of tests to discriminate the true state of subjects (e.g. health versus disease), and are used extensively in clinical epidemiology for the assessment of diagnostic ability of biomarkers (Hanley and McNeil 1982; Cook 2007; Hajian-Tilaki 2013). ROC curves represent the true-positive rate and false-positive rate associated with a particular test value. The area under the curve (AUC), or c-statistic, of the ROC curve is used as a metric to compare different ‘tests’. An AUC value close to 1 indicates an excellent diagnostic test, a curve that lies close to the diagonal (AUC = 0.5) has no information content and therefore no diagnostic utility. Although MRM-profiling is intended to assist biomarker discovery, the information can also be used to launch mechanistic studies – as once discriminatory molecules are identified investigators can then explore why they are different between the groups.

**Studies to test the efficacy of the proposed approach for discovery of fertility biomarkers**

Our overall aim is to develop a practical approach to identify replacement gilts with high potential for sow longevity. It appears that early nutritional environment, in particular colostrum exposure, affects long-term fertility in swine, with studies conducted by Bartol and Bagnell group clearly demonstrating that colostrum exposure versus milk replacer feeding resulted in distinct developmental trajectories of uterine glands in swine (Bagnell et al. 2008; Bartol et al. 2009; Chen et al. 2011; Frankshun et al. 2012; Rahman et al. 2016). Our laboratory independently confirmed these findings after our protocol was reviewed and approved by Purdue University’s Institution Animal Care and Use Committee. In our study, median-sized female piglets were either allowed to suckle dam colostrum (COL; n = 3) or immediately removed from the dam upon delivery and bottle-fed milk replacer every 2 h the first 48 h post-partum (REP; n = 3). REP gilts were then returned to the litter until postnatal day 14 when they were euthanized. The uterus was removed and 2 cm of each horn beginning at bifurcation from the body of the uterus was dissected out and plunged in liquid nitrogen. Total RNA was isolated from one horn from each animal and Q-PCR analysis found expression of the genes VEGFA and BCL2 were decreased by 43% and 47%, respectively, in uteri of gilts fed formula the first 48 h post-partum versus those that were allowed to suckle dam’s colostrum (unpublished data). These findings were consistent with previous reports (Frankshun et al. 2012; Bartol et al. 2013), and thus supported the use of this model system to establish two groups with different reproductive potential based on colostrum exposure.

With the validation of the model system, we next asked whether MRM-profiling of vaginal lipidome can distinguish between gilts that suckled colostrum and those that were fed milk replacer. In particular, swab samples were obtained from 2-day-old piglets immediately before and after euthanasia by inserting a pap brush (EndoCervex-Brush®, Rovers medical devices, The Netherlands) into the vagina and rotating device 360°against the surface of the tract. Pap brushes were then
placed in sterile conical, and transported on ice to a lab where they were stored at −20°C. After swabs were obtained, gilts were dissected to expose their reproductive tract. The most anterior region of the vagina was determined by palpation of the cervix, and the area of the vagina extending 2 cm towards the vulva was removed and plunged into liquid nitrogen. After sample thawing, 500 µL of water was added to the tube containing the pap brush and gentle mixing was used to remove biological material from the brush. The sample homogenate was collected and submitted to lipid extraction using the Bligh and Dyer method (Bligh and Dyer 1959), by which phase separation is performed using CHCl₃/MeOH/H₂O (1:2:0.8). The combined organic fractions were centrifuged, and then the bottom phase was transferred to a new tube and evaporated. Dried lipid extracts were stored at −20°C until MS analysis.

For the discovery phase of MRM-profiling, dried lipid extracts were diluted in acetonitrile (ACN)/methanol/ammonium acetate 300 mM at 3:6.65:0.35 volume ratios) and 8 µL was directly delivered through a micro-autosampler (G1377A) to a QQQ6410 triple quadrupole mass spectrometer (Agilent Technologies, San Jose, CA) operated in the positive ion mode, equipped with a Jet stream ESI ion source. The solvent pumped between injections was ACN + 0.1% formic.

For the MRM-discovery step, lipid extracts of vaginal tissue from gilts that suckled colostrum and those that were fed milk replacer were pooled with extracts from swab samples (swabs and tissues from 11 animals in total). Over 1400 molecular features related to various lipid classes were obtained from the discovery phase of the analysis (data not shown). These molecules informed us which precursor ion and neutral loss scans to use in the screening phase of MRM-profiling which was performed on individual tissue and swab samples from gilts fed with colostrum (COL; n = 3) and those that were fed milk replacer (REP; n = 3). In particular, based on PCA and ROC curve analysis of the >1400 molecular features (MRMs), 126 molecular features were selected for the screening phase of MRM-profiling. Data from the MRM-screening phase profiles were up-loaded into MetaboAnalyst 3.0 for analysis using PCA, partial least square-discriminant analysis (PLS-DA) and ROC analysis (Figures 2–6; Table 1).

Hierarchical cluster analysis (illustrated in Figure 1 with dendogram above heat map in screening phase statistics) found two distinct groups across swab (indicated by red and royal blue boxes clustered to the left) and tissue (indicated by green and cyan blue clustered primarily together) samples. Swab samples had, in general, a higher lipid content than the tissue samples. This differential distribution likely reflected the sample composition. Vaginal swabs captured luminal cells and the associated secretions. Tissue samples were composites of epithelial, stromal and muscle layers of the reproductive tract. Swab and tissue samples were thus considered separately for analysis of treatment effects on reproductive tract lipidomes.

ROC analysis of screening data of tissues (Figure 2) from COL (n = 3; with three technical replicates) and REP (n = 3; with three technical replicates) found four MRM profiles (parent and
Fragment m/z; Table 1 tentative attribution) that discriminated between the two groups. When these four MRM profiles were combined (Figure 3), the AUC for ROC analysis increased from ∼0.8 to 0.88, supporting that when these lipids are combined they make good potential biomarkers that distinguish between groups. Sensitivity (true-positive rate) and specificity (true-negative rate) analysis within this limited sample size found COL classified correctly at 77.8% (7 out of 9) and REP at 66.7% (6 out of 9). These findings are consistent with hierarchical cluster analysis and relative amounts across the samples (Figure 3(C)).

ROC curve analysis of swab samples taken pre- and post-mortem from posterior and anterior regions of the vagina (2–3 samples per animal analysed) was used to identify the four most distinguishing MRM profiles between COL (n = 3) and REP (n = 3) animals (Figure 4). When these four features were combined for further discriminatory analysis, the AUC was found to be 0.8 (Figure 5(A)), indicating a good potential set of biomarkers. The sensitivity (true-positive rate) of this set of markers for calling COL samples was 82.3% (14 out of 17) and specificity for COL (true-negative rate) was 58.8% (10 out of 17; Figure 5(B)). These data did not take into account the regions of the vagina from which samples were taken. Hierarchical cluster analysis showed two relatively distinct groups (Figure 5(C)). Post-hoc analysis with vaginal tissue region as a factor found that the area from which vagina swabs were taken (posterior versus anterior) likely affected the MRM profile (data not shown). This finding is consistent with the distinct histomorphic differences in the anterior and posterior vagina. The length of commercial pap brushes used resulted in samples that overlapped the posterior and anterior regions in pre-mortem samples taken from gilts on postnatal day 2. Post-mortem samples allowed for better relative sampling of posterior and anterior regions of the vagina, but small sample size limited the power of the study. Future studies will focus on more specifically isolating the region from which the swab is taken in the vagina (most probably anterior vagina), and applying techniques like vaginal impedance measures (Rezac et al. 2003) to better identify the regions.

Discriminant analysis (PLS-DA) of tissue and swab screening profiles was also performed (Figure 6). Partial least squares (PLS) regression is a technique that generalizes and combines features from PCA and multiple regression analysis, and is used to predict a set of dependent variables from a (very) large set of independent variables (i.e., predictors). Visualization of the PLS-DA data shows distinct groupings of samples, primarily based on treatment (COL versus REP) in both tissue and swab screening profile analyses. Thus, despite limitations of the

![Figure 3](image_url). Multivariate ROC curve analysis of vaginal tissue samples using the four molecular features with the highest AUC (>0.8). (A) ROC curve showing AUC = 0.88; (B) probability view graph where three REP samples out of nine and 2 COL samples out of nine have been misclassified (red and blue arrows, respectively); and (C) heat map and cluster analysis of relative amounts for the four selected molecular features.
Figure 4. Screening phase results for vaginal swab samples. ROC curves obtained for individual molecular features in the screening phase and distribution of COL and REP samples according to the thresholds (red lines) set for higher specificity and sensitivity. Table 1 lists tentative attributions.

Figure 5. Multivariate ROC curve analysis of vaginal swab samples using the four molecular features with the highest AUC (>0.8). (A) ROC curve showing AUC = 0.80; (B) probability view graph where 7 REP samples out of 17 and 3 COL samples out of 17 have been misclassified; and (C) heat map and cluster analysis of relative amounts for the four selected molecular features.
exploratory work, which included small sample sizes and the fact that molecular features selected for the screening phase were based on composite vaginal tissue swab samples from anterior and posterior regions of the vagina, MRM profiles of vaginal swabs indicated that groups of gilts with potentially distinct reproductive competence based on colostrum exposure could be distinguished. Together these findings support further exploration of using MRM-profiling to identify biomarkers in gilts predictive of long-term fertility in swine.

If our future studies further support our hypothesis, and biomarkers predictive of sow reproductive competence are identified in the lipidome of the vaginal swab samples taken at weaning, we envision development of a quick diagnostic test. Biomarkers predictive of fertility outcomes may facilitate quick, applied research capable of producing farm-level management changes. Early identification of superior gilts using biomarkers has the potential to save the industry millions of dollars in feed and management costs of subfertile animals. Additionally, biomarkers can be used as a research tool to investigate the effects of bioactive compounds in colostrum/milk that can enhance reproductive potential, as well as identify the best or most cost effective neonatal supplements (e.g. fats, hormones, etc.) to improve lifetime production.

**Disclosure statement**

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