ABSTRACT: Histone modification is a well-known epigenetic mechanism involved in regulation of gene expression; however, it has been poorly studied in adipose tissues of the pig. Understanding the molecular background of adipose tissue development and function is essential for improving production efficiency and meat quality. The objective of this study was to identify the association between histone modification and the transcript level of genes important for lipid droplet formation and metabolism. Histone modifications at the promoter regions of 6 genes (SNAP23, BSCL2, COPA, LEP, SCD, and FABP4) were analyzed using a chromatin immunoprecipitation assay. Two modifications involved in activation of gene expression (acetylation of H3 histone at lysine 9 and methylation of H3 histone at lysine 4) as well as methylation of H3 histone at lysine 27, which is known to be related to gene repression, were examined. The level of histone modification was compared with transcript abundance determined using real-time PCR in tissue samples (subcutaneous fat, visceral fat, and longissimus dorsi muscle) derived from 3 pig breeds significantly differing in fatness traits (Polish Large White, Duroc, and Pietrain). Transcript levels were found to be correlated with histone modifications characteristic to active loci in 4 of 6 genes. A positive correlation between histone H3 lysine 9 acetylation modification and the transcript level of SNAP23 \( r = 0.53, P < 4.8 \times 10^{-3} \), BSCL2 \( r = 0.34, P < 0.02 \), and LEP \( r = 0.43, P < 1.0 \times 10^{-5} \) genes was observed. The histone H3 lysine 4 trimethylation modification correlated with transcripts of SNAP23 \( r = 0.64, P < 4.6 \times 10^{-4} \) and COPA \( r = 0.37, P < 0.01 \) genes. No correlation was found between transcript level of all studied genes and histone H3 lysine 27 trimethylation level. This is the first study on histone modifications in porcine adipose tissues. We confirmed the relationship between histone modifications and expression of key genes for adipose tissue accumulation in the pig. Epigenetic modulation of the transcriptional profile of these genes (e.g., through nutritional factors) may improve porcine fatness traits in future.

Key words: adiposity, chromatin immunoprecipitation, epigenetics, fatness, histone H3 acetylation, histone H3 methylation

INTRODUCTION

Modern pig breeding programs focus on improving pork quality, and special attention is paid to fat content and composition (Pena et al., 2016). Phenotypic variations of fat deposition are known to be significantly influenced by genetic and environmental factors as well as their interactions. Over the past years, many efforts have been made to identify the genetic background of fatness in pigs (Switonski et al., 2010; Stachowiak et al., 2016). However, the identified gene polymorphisms explain only part of the phenotypic variation of the trait. Several studies have shown an important role of epigenetics in development of human obesity (Martínez et al., 2014; van Dijk et al., 2015a,b). Therefore, epigenetic mechanisms have received increasing interest from the livestock sciences. It is believed that they are responsible for a portion of the missing variation in production traits (Ibeagha-Awemu and Zhao, 2015).
Among epigenetic mechanisms, only DNA methylation has been widely studied in relation to fat deposition and fatty acid composition in pigs (Li et al., 2012a,b; Zhang et al., 2016). Another important process—post-translational histone modification—is poorly recognized in this species. It has been well established that covalent attachment of chemical groups onto histones such as acetylation, methylation, phosphorylation, and ubiquitination regulate gene transcription by affecting chromatin structure (Kouzarides, 2007). Most studies concerning the role of histone modifications on expression of genes important for adipogenesis have been performed on cell lines and mouse models (Okamura et al., 2010; Zhang et al., 2012; Zhou et al., 2014). To date, data regarding the role of these epigenetic marks in controlling transcription of genes in adipose tissues is lacking.

The aim of this study was to analyze histone modifications at promoter regions of key genes important for accumulation of adipose tissue in relation to transcript abundance of these genes. Altogether, 6 genes involved in lipid droplet formation (SNAP23- synaptosomal-associated protein 23, BSCL2- Berardinelli-Seip congenital lipodystrophy 2 - seipin, COPA- coatamer protein complex α) and lipid metabolism (LEP- leptin, SCD- Stearoyl-CoA Desaturase, FABP4- Fatty Acid Binding Protein 4) were selected. The following histone modifications were studied: histone H3 lysine 9 acetylation (H3K9Ac), histone H3 lysine 4 trimethylation (H3K4Me3), and histone H3 lysine 27 trimethylation (H3K27Me3). The chromatin immunoprecipitation (ChIP) approach was applied to tissue samples (subcutaneous fat, visceral fat, and longissimus dorsi muscle) from 3 pig breeds that significantly differed in fatness traits (Polish Large White, Duroc, and Pietrain).

**MATERIALS AND METHODS**

**Animals**

All animal procedures were approved by the Local Ethical Commission on Experiments on Animals at the Poznan University of Life Sciences, Poznan, Poland (number 57/2012). The study was performed on unrelated gilts representing 3 pig breeds differing in fatness traits: Polish Large White (n = 5), Duroc (n = 5), and Pietrain (n = 5). The gilts were reared under identical housing and feeding conditions at a Pig Testing Station (Pawlowice, Poland). They were individually fed ad libitum with a commercial fodder until they reached 100 to 105 kg in BW, and then they were fasted for 24 h prior to slaughtering. Tissue samples of subcutaneous fat, visceral fat, and longissimus dorsi muscle were collected immediately after slaughter. The materials were snap-frozen in liquid nitrogen and stored at ~80°C until RNA and chromatin isolation.

**Gene Expression Analysis**

Total RNA from frozen samples was extracted using a TissueLyser LT homogenizer (Qiagen GmbH, Hilden, Germany) and TriPure Isolation Reagent RNA (Roche – Applied Science, Indianapolis, IN) according to the manufacturers’ instructions with some modifications described earlier by Kociucka et al. (2016b). Concentration and purity of isolated RNA were determined using absorbance measurements at 260 and 280 nm on a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). An aliquot of 2 µg of RNA was reverse transcribed using a Transcriptor High Fidelity cDNA Synthesis kit (Roche – Applied Science). The semi-quantitative transcript level analysis was performed in duplicate using a Fast Start DNA MasterPlus SYBR Green I kit (Roche – Applied Science) on a LightCycler 480 II (Roche – Applied Science). The relative transcript level was calculated after correction using the transcript levels of 2 reference genes: beta-actin (ACTB) and peptidylprolyl isomerase A (PPIA). The primer sequences and PCR details are given in Supplementary Table S1 (see the online version of the article at http://journalofanimalscience.org).

**Chromatin Immunoprecipitation Assay**

Samples of adipose and muscle tissues were ground in liquid nitrogen, resuspended in PBS, and cross-linked in 1.5% formaldehyde for 15 min at room temperature. The cross-linking reaction was stopped by addition of glycine to a final concentration of 125 mM and subsequent incubation for 10 min at room temperature. Next, samples were centrifuged at 4,000 × g and the tissue pellet was washed 2 times with ice-cold PBS. Protease inhibitors were included in all solutions used. Then, the tissue pellet was resuspended in lysis buffer (5 mM piperazine-N,N’-bis(ethanesulfonic acid), pH 8.0; 80 mM KCl; and 1% IGEPAL [Sigma-Aldrich, St. Louis, MO]) and homogenized using a TissueLyser LT homogenizer (Qiagen GmbH). From this step, buffers included in a Low Cell ChIP kit (Diagenode s.a., Liège, Belgium) were used according to the manufacturer’s instructions. Buffer B was added to the lysate, and chromatin fragmentation was performed by sonication using a Bioruptor Plus (Diagenode s.a.). The appropriate length of sonicated chromatin (between 200 and 600 bp) was determined using a BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA). The following antibodies were used in the immunoprecipitation reactions: anti-H3K4me3, anti-H3K9ac, and anti-H3K27me3 (Diagenode s.a.). Their
specificity has been confirmed using Western blotting. Rabbit IgG was included as a negative control. Protein A–coated magnetic beads (Diagenode s.a.) with bound antibodies were prepared according to the manufacturer’s instructions and were incubated with shared chromatin samples overnight at 4°C with constant rotation. Thereafter, beads were washed using Buffer A and Buffer C from the Low Cell ChIP kit (Diagenode s.a.) and captured using a magnetic rack. Obtained immunoprecipitated complexes as well as input samples (sheared chromatin not subjected to immunoprecipitation) were used for DNA isolation. Samples were treated with Proteinase K and Buffer DIB from the Low Cell ChIP kit. The resulting DNA was quantified using real-time quantitative PCR (qPCR) using LightCycler FastStart DNA Master Plus SYBR Green I (Roche – Applied Science). Primers for the qPCR were designed in the genomic region located upstream of the transcription start site using Primer3 software (http://frodo.wi.mit.edu/primer3/; Supplementary Table S1 [see the online version of the article at http://journalofanimalscience.org]). The efficiency of the ChIP for a particular genomic locus was calculated from qPCR data using the percent input method.

**Statistical Analysis**

The linear relationship between histone modification level (log scale) and transcript level (log scale) was measured and tested with Pearson product moment correlation. To exclude false correlations, variables were adjusted for breed and tissue as estimated in a linear model. The adjusted transcript level was regressed on the histone modification level to derive the coefficient of determination ($R^2$). All calculations were performed in the R statistical environment (R Core Team, 2017).

## RESULTS AND DISCUSSION

To determine if histone modifications correlate with transcriptional activity, in the first step, we analyzed the relative transcript levels of 6 genes ($SNAP23$, $BSCL2$, $COPA$, $LEP$, $SCD$, and $FABP4$) in 2 adipose tissues (subcutaneous and visceral) and 1 muscle tissue (longissimus dorsi) from 3 pig breeds. The same samples were used to measure the level of histone modifications of the promoter regions of the studied genes. A ChIP assay with specific antibodies against histone H3 acetylation and methylation was performed. We found that histone modification and gene expression levels were very well correlated in 4 of the 6 studied genes (Table 1). Two modifications that are known to be associated with transcriptionally active loci have shown positive correlation with $SNAP23$ gene expression: H3K9Ac ($r = 0.53, P < 4.8 \times 10^{-3}$) and H3K4Me3 ($r = 0.64, P < 4.6 \times 10^{-4}$; Fig. 1). Additionally, a positive correlation between H3K9Ac modification and the transcript level of the $BSCL2$ ($r = 0.34, P < 0.02$) and $LEP$ ($r = 0.43, P < 1.0 \times 10^{-3}$) genes was observed. The H3K4me3 modification correlated ($r = 0.37, P < 0.01$) with expression of the $COPA$ gene. No correlation was found between the transcript level of all studied genes and the H3K27Me3 level.

Cell type–specific gene expression is regulated by complex interactions between the DNA sequence and transcription factors as well as DNA methylation and histone modifications (Natarajan et al., 2012). To date, in the pig, the role of histone modifications on gene expression regulation is poorly recognized. Only a few studies have focused on analysis of histone modifications of single genes in the pig, for example, $MSTN$ in muscle (Liu et al., 2011) or $HMGCR$ and $CYP7A1$ in the liver (Cong et al., 2012; Cai et al., 2014). This study is the first to examine histone modifications in porcine adipose tissues. We selected 3 modifications of histone H3 with differing impacts on transcription. The acetylation of histone H3 is mediated by histone acetyltransferases and induces an open chromatin conformation and promotes transcription, whereas histone deacetylases reverse histone acetylation, resulting in a more compact chromatin and repression of transcription (Wang et al., 2009). Histone methylation has both positive and negative effects on transcription depending on the site of modification and, in the case of lysine methylation, is mediated by histone lysine methyltransferase (Bannister and Kouzarides, 2011).

We found correlations between H3K9Ac modification and the transcript levels of 3 genes and between

| Gene   | H3K27Me3 | H3K9Ac | H3K4Me3 |
|--------|----------|--------|---------|
| Function | Name  | Active | Repressive | Active |
| Lipid  | SNAP23  | 0.64   | 0.64     | 0.64    |
| formation | BSCL2 | 0.25   | 0.25     | 0.25    |
| COPA   | 0.73    | 0.73   | 0.73     | 0.73    |
| Lipid  | SCD     | 0.01   | 0.01     | 0.01    |
| metabolism | LEP | < 0.01 | < 0.01 | < 0.01 |
| FABP4  | < 0.01  | < 0.01 | < 0.01   | < 0.01  |

1 H3K27Me3 = histone H3 lysine 27 trimethylation; H3K9Ac = histone H3 lysine 9 acetylation; H3K4Me3 = histone H3 lysine 4 trimethylation.

2 NS = nonsignificant.
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H3K4Me3 and the expression of 2 genes. These specific modifications are known to be correlated with gene activation and are enriched at the promoters, around the transcription start and enhancers (Ge, 2012). Among 4 genes showing transcript level/histone modification correlation, 3 are involved in lipid droplet formation. Our previous study indicated that SNAP23, BSCL2, and COPA may be considered interesting candidates involved in adipose tissue growth in the pig (Kociucka et al., 2016a). The most interesting study result concerns the SNAP23 gene for which prominent correlations ($r = 0.5$ and $r = 0.6$) for 2 modifications characteristic for transcription activation were observed. The SNAP23 gene encodes lipid droplet–associated protein, and its expression is related to the size of adipocytes in porcine adipose tissues (Kociucka et al., 2016b). Only 1 gene (LEP) from these involved in lipid metabolism has shown correlation with histone modification. The leptin is a well-known adipokine, and its expression is positively associated with the extent of adiposity (Reiter et al., 2007). Expression of this gene has also been revealed to be regulated by epigenetic mechanisms, including histone modifications, in mice with diet-induced obesity (Shen et al., 2014). Trimethylation of histone H3 lysine 27 is a known repressive mark of transcription and is mainly found at the promoter area of inactive genes (Kooistra and Helin, 2012). Because the studied genes were expressed in the analyzed tissues, we observed a tendency for a negative correlation only between the level of transcript and the level of this modification.

Different results were obtained when histone modifications were analyzed in terms of sex differences. It has been shown that the pattern of H3K9Me2 modification in human lymphocytes or monocytes was irrespective of age or gender but was highly specific to cell type (Miao et al., 2008). In contrast, the H3K27Me3 mark has been recognized as important for regulation of sex-biased gene expression in the mouse liver (Sugathan and Waxman, 2013). In this study we performed the experiment on gilts kept at the Pig Testing Station. In the station a sire’s breeding value is evaluated on daughters exclusively. With the limited sample size available for this study, a statistical model with a restricted source of variation (the same age, sex, feeding, and BW at slaughter) was considered the most efficient. However, we think that further studies on a potential sex-biased gene expression in porcine adipose tissues, controlled by histone modifications, are needed.

Several epigenetic marks have been recognized to be possibly reversed or modified by environmental factors, including nutrition (Choi and Friso, 2010; van Dijk et al., 2015b). A variety of nutrients or bioactive food components have been shown to modulate activity of enzymes such as histone deacetylases, histone acetyltransferases, and DNA methyltransferase (Busch et al., 2015; Vahid et al., 2015). This raises the possible influence of expression of genes important for fat deposition. For example, leptin gene expression in mice has been shown to be regulated by $n$-3 PUFA through at least partial histone modifications (Shen et al., 2014). Other factors, such as exercise or surgery interventions, have also been shown to have the ability to influence the epigenome in human studies (Barrès and Zierath, 2016). One of many examples, the SCD gene, also deserves mention; changes in the methylation level of its promoter were reported in morbidly obese subjects after bariatric surgery (Morcillo et al.,

![Figure 1](https://academic.oup.com/jas/article-abstract/95/10/4514/4772007/4517)

Figure 1. The relationship between transcript level and histone H3 lysine 9 acetylation (H3K9Ac; A) and histone H3 lysine 4 trimethylation (H3K4Me3; B) enrichment for the SNAP23 gene. Pearson correlation coefficient ($r$), 95% confidence interval (CI), $P$-value for test of no correlation ($P$), coefficient of determination ($R^2$), and regression line are shown.
Furthermore, it can be anticipated that in livestock, studies on modulation of epigenetic modifications aimed at improving production traits will develop. Our study is a first step in comprehending the role of post-translation-al histone modifications on the transcriptional profile of genes crucial for fat cell metabolism, thereby possibly affecting fatness in pigs.

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