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Transcriptional and epigenetic regulation of in vitro adipogenic differentiation of porcine Mesenchymal Stem Cells (MSCs)

Doctoral dissertation in the discipline of Biological Sciences

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Poznań, 2026

Acknowledgments

First and foremost, I would like to express my deepest gratitude to Prof. Dr. hab. Izabela Szczerbal, my supervisor, with whom I had the privilege of conducting the entire course of my doctoral studies. I am sincerely grateful for the invaluable knowledge and experience shared with me throughout this journey. Her patience, guidance, encouragement, and constant support have contributed immensely not only to this dissertation but also to my scientific perspective and personal development. I have always felt fortunate to work with such an inspiring academic and, above all, one of the kindest and most remarkable people I have ever met.

I would also like to express my sincere gratitude to Adrianna Bilińska and Jędrzej Rożynek for their invaluable contributions to the experiments conducted throughout this study. Their support, assistance, and dedication during the rearing and execution of the experimental trials played a crucial role in the completion of this dissertation, and without their help, this work would not have been possible.

Since the very first day I arrived in Poland, I have been fortunate to meet many wonderful people who made this journey both easier and more meaningful. In this regard, I would like to sincerely thank my friends from Room 44, especially Michał Antkowiak, Jakub Wozniak, and all the others who welcomed me as one of their own and supported me in every possible way. I would also like to express my special gratitude to Weronika Loba-Pasternak, who became an honorary part of Room 44.

I would also like to thank all the wonderful people in the Department of Genetics and Animal Breeding, who warmly welcomed me from the very beginning and made me feel comfortable and supported throughout my time in the department.

Finally, and above all, I owe my deepest gratitude to my mother, Figen Aksoy, my father, Necdet Aksoy, and my family, who have never withheld their love, support, and encouragement from me. Knowing that they were always by my side gave me strength throughout every stage of this journey.

This doctoral dissertation is based on three published scientific articles

1. **Aksoy, M. O**.*, Bilinska, A.*, Stachowiak, M., Flisikowska, T., & Szczerbal, I. (2024). Deciphering the role of the *SREBF1* gene in the transcriptional regulation of Porcine adipogenesis using CRISPR/Cas9 editing. *International Journal of Molecular Sciences*, 25(23), 12677. https://doi.org/10.3390/ijms252312677 MNiSW points= 140, 5-year Impact Factor= 5.7

2. **Aksoy, M. O.**, Rozynek, J., Stachowiak, M., & Szczerbal, I. (2026). The role of the *CEBPB* gene in porcine adipogenesis: a study using CRISPR/Cas9-edited mesenchymal stem cells. *Journal of Applied Genetics*, 67, 447-486. https://doi.org/10.1007/s13353-025-01030-x MNiSW points=140, 5-year Impact Factor= 2.0

3. **Aksoy, M. O**., Wozniak, J., Stachowiak, M., & Szczerbal, I. (2026). Dynamic Histone Modification Patterns in Key Transcription Factor Genes During Porcine Adipogenesis. *Genes*, 17(5), 521. https://doi.org/10.3390/genes17050521 MNiSW points= 100, 5-year Impact Factor= 3.2

This PhD project was funded by National Science Center (Poland), NCN OPUS Project number 2018/29/B/NZ2/00956, titled "**The Application of CRISPR-Cas9 Technology to Elucidate whether Changes in Nuclear Architecture During Adipogenesis are a Cause or a Consequence?**" (Leader: Prof. Dr. hab. Izabela Szczerbal)

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1. List of Abbreviations

AD-MSC — Adipose-derived mesenchymal stem cell

ADSC — Adipose-derived stem cell

Cas9 — CRISPR-associated protein 9

cDNA — Complementary DNA

CEBPA — CCAAT/enhancer-binding protein alpha

CEBPB — CCAAT/enhancer-binding protein beta

CEBPD — CCAAT/enhancer-binding protein delta

CCND1 — Cyclin D1

CCAAT — Cytosine–Cytosine–Adenine–Adenine–Thymine

ChIP-qPCR — Chromatin immunoprecipitation followed by quantitative PCR

CpG — Cytosine–phosphate–guanine dinucleotide

CRISPR — Clustered regularly interspaced short palindromic repeats

crRNA — CRISPR RNA

DAPI — 4′,6-diamidino-2-phenylindole

DNA — Deoxyribonucleic acid

DMEM — Dulbecco's Modified Eagle Medium

FABP4 — Fatty acid-binding protein 4

FBS — Fetal bovine serum

FDR — False discovery rate

FGF-2 — Fibroblast growth factor 2

GATA2 — GATA binding protein 2

GFP — Green fluorescent protein

gRNA — Guide RNA

HDR — Homology-directed repair

H3K9ac — Histone H3 lysine 9 acetylation

H3K27ac — Histone H3 lysine 27 acetylation

H3K27me3 — Histone H3 lysine 27 trimethylation

H4K8ac — Histone H4 lysine 8 acetylation

H4K20me3 — Histone H4 lysine 20 trimethylation

HDR — Homology-directed repair

IgG — Immunoglobulin G

ITS — Insulin–transferrin–selenium

MANOVA — Multivariate analysis of variance

MCE — Mitotic clonal expansion

MCM2 — Minichromosome maintenance complex component 2

MSC — Mesenchymal stem cell

NEAA — Non-essential amino acids

NHEJ — Non-homologous end joining

PBS — Phosphate-buffered saline

PCNA — Proliferating cell nuclear antigen

PPIA — Peptidylprolyl isomerase A

PPARG — Peroxisome proliferator-activated receptor gamma

PCR — Polymerase chain reaction

qPCR — Quantitative polymerase chain reaction

QTL — Quantitative trait loci

RNA — Ribonucleic acid

RPL27 — Ribosomal protein L27

RT-qPCR — Reverse transcription quantitative PCR

SREBF1 — Sterol regulatory element-binding transcription factor 1

SREBF1a — Sterol regulatory element-binding transcription factor 1a

SREBF1c — Sterol regulatory element-binding transcription factor 1c

WBP — Polish Large White breed

2. Streszczenie

Adipogeneza jest złożonym i ściśle regulowanym procesem biologicznym, odgrywającym kluczową rolę w utrzymaniu homeostazy energetycznej oraz rozwoju otyłości. Powstawanie adipocytów jest szczególnie istotne dla świń, ponieważ tkanka tłuszczowa silnie wpływa na tempo wzrostu, bilans energetyczny, przeżywalność noworodków oraz cechy jakości mięsa, które są bardzo ważne z punktu widzenia produkcji zwierzęcej. Chociaż zidentyfikowano wiele kluczowych czynników transkrypcyjnych uczestniczących w adipogenezie, współdziałanie mechanizmów transkrypcyjnych i epigenetycznych regulujących ten proces pozostaje nadal niewystarczająco poznane, szczególnie u gatunków gospodarskich, takich jak świnia.

Głównym celem niniejszej pracy było poznanie molekularnych mechanizmów regulujących adipogenezę u świń, ze szczególnym uwzględnieniem roli kluczowych czynników transkrypcyjnych, ich wzajemnych interakcji oraz modyfikacji histonów związanych z różnicowaniem adipocytów. Analizy transkrypcyjne i epigenetyczne przeprowadzono z wykorzystaniem świńskich mezenchymalnych komórek macierzystych (MSC) jako modelu in vitro. W celu poznania roli ważnych genów kodujących czynniki transkrypcyjne adipogenezy - *SREBF1* i *CEBPB* zastosowano edycję genomu za pomocą metody CRISPR/Cas9. Wpływ wprowadzonych delecji genów na różnicowanie adipocytów oceniano poprzez analizę ekspresji głównych markerów adipogenezy, obejmujących geny *PPARG, GATA2, CEBPA, CEBPD* i *FABP4*. Dodatkowo, oceniono regulację epigenetyczną poprzez analizę wybranych aktywujących i represyjnych modyfikacji histonów, tj. H4K20me3, H4K8ac oraz H3K9ac, w obrębie loci genów *PPARG, GATA2, CEBPA* oraz *CEBPB.*

W badaniach zastosowano szereg metod eksperymentalnych, obejmujących hodowlę in vitro MSC, indukcję różnicowania w kierunku adipocytów, edycję genomu z wykorzystaniem techniki CRISPR/Cas9, generowanie komórek z homozygotycznymi i heterozygotycznymi delecjami, PCR i sekwencjonowanie DNA w celu potwierdzenia obecności mutacji, analizę akumulacji kropli lipidowych za pomocą immunobarwienia, izolację RNA, ilościową reakcję PCR po odwrotnej

transkrypcji (RT-qPCR) do analizy ekspresji genów, immunoprecypitację chromatyny połączoną z ilościowym PCR (ChIP-qPCR) do analizy modyfikacji histonów oraz analizę korelacji pomiędzy aktywnością transkrypcyjną a modyfikacjami chromatyny.

Uzyskane wyniki wykazały, że zaburzenie funkcji izoformy *SREBF1c* istotnie zmieniało ekspresję kluczowych genów adipogennych, w tym prowadziło do obniżenia ekspresji genów *PPARG*, *CEBPA*, *CEBPD* oraz *FABP4*, oraz wzrost ekspresji genu *CEBPB*, co ostatecznie skutkowało zahamowaniem adipogenezy i ograniczeniem akumulacji kropli lipidowych. Po raz pierwszy wykazano kluczową rolę izoformy *SREBF1c* w różnicowaniu adipocytów u świń oraz zidentyfikowano potencjalną współzależność regulacyjną pomiędzy *SREBF1c* i *CEBPB*.

W przypadku genu *CEBPB* edycja genomu metodą CRISPR/Cas9 doprowadziła do powstania zarówno homozygotycznych, jak i heterozygotycznych delecji w obrębie regionu promotorowego. Oba typy modyfikacji całkowicie blokowały różnicowanie adipocytów oraz wpływały na zdolność proliferacyjną świńskich mezenchymalnych komórek macierzystych, co potwierdzono zmienionymi profilami ekspresji genów związanych z proliferacją, takich jak *CCND1*, *MCM2* i *PCNA*. Wyniki te wykazały kluczową rolę *CEBPB* nie tylko w adipogenezie, lecz także w regulacji proliferacji komórkowej.

Ponadto analizy epigenetyczne ujawniły dynamiczne i zależne od etapu różnicowania zmiany aktywujących (H3K9ac i H4K8ac) oraz represyjnych (H4K20me3) modyfikacji histonów w obrębie loci adipogennych genów *PPARG*, *GATA2*, *CEBPA* i *CEBPB*. Analiza modyfikacji histonów specyficznych dla regionów promotorowych i eksonowych dostarczyła szczegółowych informacji dotyczących przebudowy chromatyny w różnych regionach genów oraz związku z regulacją transkrypcji podczas różnicowania adipocytów. Zaobserwowane profile modyfikacji histonów były specyficzne zarówno dla poszczególnych genów, jak i regionów genomowych, a ich zależność z aktywnością transkrypcyjną pokrywała się jedynie częściowo, co wskazuje na złożoną epigenetyczną kontrolę adipogenezy u świń.

Podsumowując, niniejsza praca wykazała, że adipogeneza u świń jest regulowana przez złożone interakcje pomiędzy kluczowymi czynnikami transkrypcyjnymi a mechanizmami epigenetycznymi kontrolującymi organizację chromatyny i ekspresję genów. Istotnym elementem badań było zastosowanie nowoczesnej technologii edycji genomu CRISPR/Cas9 w świńskich mezenchymalnych komórkach macierzystych, co stanowi skuteczne narzędzie do badań funkcji genów u gatunków gospodarskich. Uzyskane wyniki nie tylko poszerzają aktualną wiedzę na temat molekularnych mechanizmów rozwoju tkanki tłuszczowej świni, lecz także wskazują na potencjał wykorzystania w przyszłości technologii edycji genomu w hodowli zwierząt w celu uzyskania zwierząt o pożądanych cechach produkcyjnych, takich jak ulepszony skład tuszy i lepsza jakość mięsa.

3. Abstract

Adipogenesis is a complex and tightly regulated biological process that plays a central role in energy homeostasis and the development of obesity. In pigs, adipocyte differentiation is of particular interest, as adipose tissue strongly influences growth performance, energy balance, neonatal survival, and meat quality traits relevant to agricultural production. Although several key transcription factors involved in adipogenesis have been identified, the interplay between transcriptional and epigenetic mechanisms regulating this process remains insufficiently understood, especially in livestock species such as pigs.

The general aim of this study was to investigate the molecular mechanisms regulating adipogenesis in pigs, with particular emphasis on the role of key transcription factors, their regulatory interactions, and histone modifications associated with adipocyte differentiation. Transcriptional and epigenetic analyses were performed using porcine mesenchymal stem cells (MSCs) as an *in vitro* model system. CRISPR/Cas9-mediated gene editing was applied to investigate the functional roles of the key regulatory genes *SREBF1* and *CEBPB*. The effects of these gene disruptions on adipogenic differentiation were evaluated through the expression profiling of major adipogenic markers, including *PPARG*, *GATA2*, *CEBPA*, *CEBPD*, and *FABP4*. In addition, epigenetic regulation was assessed by analyzing selected activating and repressive histone modification marks, namely H4K20me3, H4K8ac, and H3K9ac, within the *PPARG*, *GATA2*, *CEBPA*, and *CEBPB* gene loci.

The study employed a range of research methods, including MSC culture, adipogenic differentiation assays, CRISPR/Cas9-mediated genome editing, generation of homozygous and heterozygous knockout cells, genomic PCR and DNA sequencing for mutation verification, analysis of lipid droplet accumulation by immunostaining, RNA isolation, reverse transcription quantitative PCR (RT-qPCR) for gene expression profiling, chromatin immunoprecipitation combined with

quantitative PCR (ChIP-qPCR) for histone modification analysis, and correlation analysis between transcriptional activity and chromatin modifications.

The obtained results demonstrated that disruption of the *SREBF1c* isoform significantly altered the expression of key adipogenic genes, including decreased expression of *PPARG*, *CEBPA*, *CEBPD*, and *FABP4*, accompanied by increased *CEBPB* expression, ultimately leading to the inhibition of adipogenesis and impaired lipid droplet accumulation. For the first time, the essential role of the *SREBF1c* isoform in porcine adipocyte differentiation was demonstrated, and a potential coregulatory relationship between *SREBF1c* and *CEBPB* was identified.

In the case of the *CEBPB* gene, CRISPR/Cas9-mediated gene editing resulted in both homozygous and heterozygous deletions within the promoter region. Both types of modifications completely blocked adipocyte differentiation and affected the proliferation capacity of porcine mesenchymal stem cells, as confirmed by altered expression patterns of proliferation-associated genes, including *CCND1*, *MCM2*, and *PCNA*. These findings demonstrated the crucial role of *CEBPB* is not only in adipogenesis but also in the regulation of cell proliferation.

Furthermore, epigenetic analyses revealed dynamic and stage-dependent changes in activating (H3K9ac and H4K8ac) and repressive (H4K20me3) histone modifications within the adipogenic loci of *PPARG*, *GATA2*, *CEBPA*, and *CEBPB*. The analysis of promoter- and exon-specific histone modifications provided detailed insight into chromatin remodeling across distinct gene regions and its relationship with transcriptional regulation during adipocyte differentiation. The observed histone modification patterns were both gene- and region-specific and only partially correlated with transcriptional activity, indicating complex epigenetic control of porcine adipogenesis.

In summary, this study demonstrated that adipogenesis in pigs is regulated by complex interactions between key transcription factors and epigenetic mechanisms controlling chromatin organization and gene expression. Importantly, the research involved the application of advanced CRISPR/Cas9-mediated genome editing in porcine mesenchymal stem cells, providing a modern and effective approach for

functional genomic studies in livestock species. These findings not only expand current knowledge of the molecular mechanisms underlying adipose tissue development but also highlight the future potential of genome editing technologies for the development of animals with desirable production traits, including improved carcass composition and meat quality.

4. Introduction

4.1. Obesity and Its Importance

Obesity is a multidimensional metabolic disease. It is widely considered as one of the top significant problems facing modern global health. Due to its fast growing number in both developed and developing countries, the World Health Organization has declared obesity to be a global epidemic. (WHO, 2021; Blüher, 2019). This increase is strongly associated with changes in lifestyle, including high-calorie diets and reduced physical activity. However, obesity cannot be explained by lifestyle factors alone. Genetic predisposition, hormonal regulation, metabolic imbalance, psychological conditions, environmental influences, and socioeconomic status all contribute to its development (Hruby & Hu, 2015; Speakman, 2013; Hill et al., 2012).

Obesity is closely linked to a wide range of chronic diseases. It is a major risk factor for type 2 diabetes, cardiovascular diseases, hypertension, and metabolic syndrome and many more (Kahn et al., 2006; Grundy, 2016) (Figure 1). Additionally, obesity is also associated with nonalcoholic fatty liver disease, osteoarthritis, obstructive sleep apnea, and several types of cancer (Lauby-Secretan et al., 2016; Haslam & James, 2005). Emerging evidence also suggests a connection between obesity and neurodegenerative diseases such as Alzheimer's disease, as well as respiratory disorders including asthma (Ng et al., 2014; Profenno et al., 2010). These conditions significantly decrease the quality of life and increase mortality rates. Furthermore, obesity can lead to systemic inflammation and organ dysfunction, making it a critical factor in the development of complex metabolic disorders (Gregor & Hotamisligil, 2011).

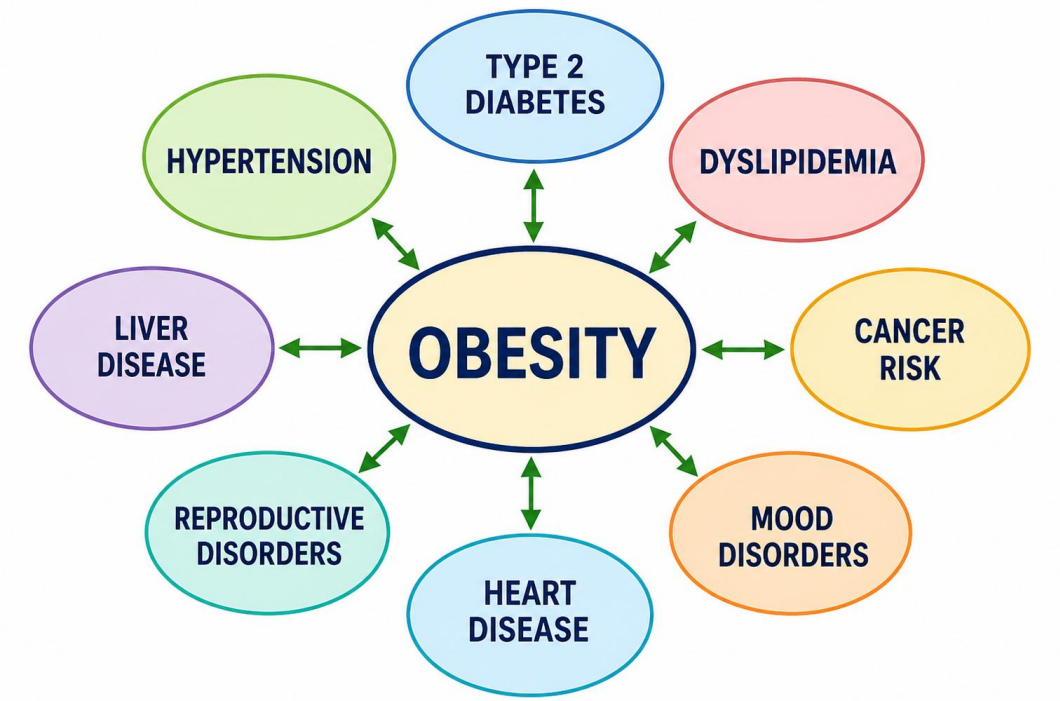

Figure 1. Schematic representation of some obesity related diseases. Obesity contributes to a wide range of metabolic and systemic complications. *Modified from* Kyrou et al., (2015).

In addition to these factors, obesity is recognized as a critical factor that plays a significant role in the onset and progression of complex metabolic disorders, as it leads to systemic inflammation and organ dysfunction (Gregor & Hotamisligil, 2011). Due to the rapidly increasing prevalence and complexity of obesity worldwide, various animal models have been, and continue to be, utilized to understand its molecular and physiological mechanisms better. While rodent models remain the most commonly used systems in obesity research, larger animal models such as pigs and dogs are becoming increasingly prevalent as model organisms in the scientific community due to their closer physiological and metabolic similarities to humans (Lutz & Woods, 2012; Spurlock & Gabler, 2008; Stachowiak et al., 2016).

Excessive accumulation of fat tissue is not only a problem for human health but also a major issue in animal production systems. In farm animals, high fat deposition is frequently associated with intensive feeding regimes aimed at maximizing growth and production. High energy diets, combined with limited physical activity in confined housing systems, promote rapid weight gain and increased adiposity (Hausman et al., 2009; D'Eath et al., 2010). In pigs, which are commonly raised under controlled feeding conditions, this can lead to metabolic imbalance and excessive fat deposition.

Fatness in pigs may have multiple negative consequences. It is associated with metabolic disorders such as insulin resistance and fatty liver, as well as cardiovascular and respiratory problems (Rauw et al., 1998; Rosen & Spiegelman, 2014). Excess body weight also contributes to musculoskeletal issues, including joint and foot disorders, which reduce mobility and welfare (D'Eath et al., 2010). In addition, immune function may be impaired, increasing susceptibility to disease. Reproductive performance is also negatively affected, leading to reduced fertility and productivity (Hausman et al. 2009). These factors directly impact economic efficiency in animal production systems through increased veterinary costs, reduced growth performance, and lower product quality (Figure 2).

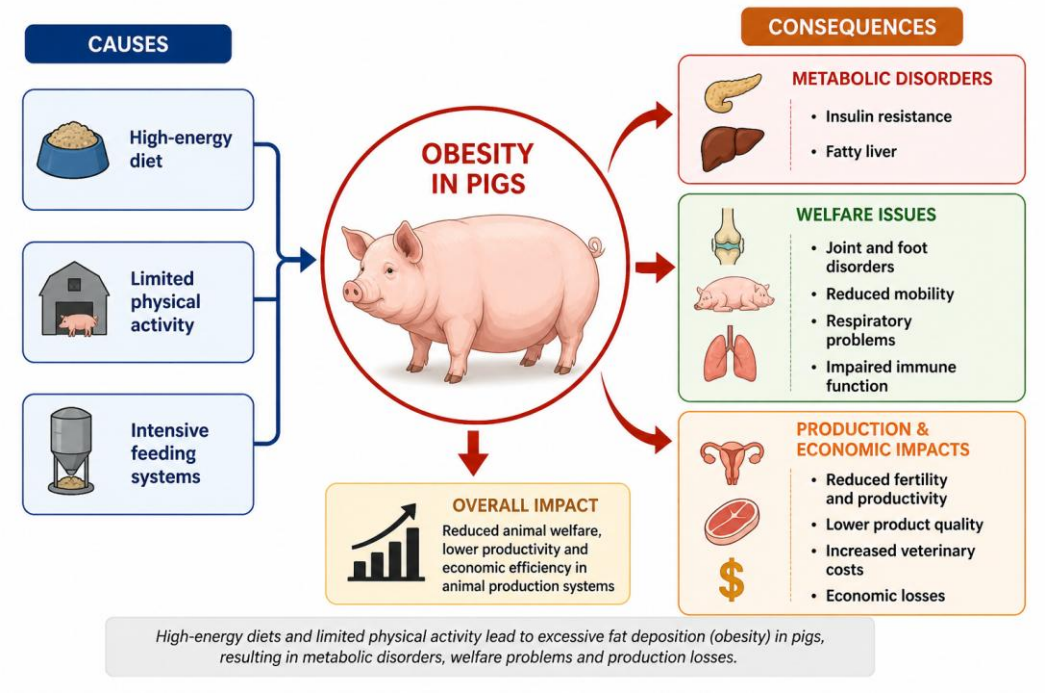

Figure 2. Schematic illustration of increased adiposity in pigs and its effects on metabolism, animal welfare, and production performance.

Despite such challenges, adipose tissue is not only a fat depot but also a key regulator of metabolic homeostasis. Fat, adipose tissue does not merely serve as a fat reservoir but also plays a pivotal role as a key regulator of metabolic homeostasis. In farm animals, adipose tissue undertakes significant physiological roles that extend beyond energy storage. The proper development and function of adipose tissue are essential for animal health, neonatal survival, reproductive performance, postnatal growth, and production efficiency (Louveau et al., 2016). Furthermore, adipose tissue functions as an endocrine organ, contributing to the regulation of metabolism, immune responses, and energy balance. Therefore, understanding not only the properties but also the development and function of adipose tissue is of paramount importance for both scientific research and for comprehending its significance in animal production. Taking the above into account, it is very, it is important to understand how adipose tissue develops and functions. At the cellular level, obesity is closely associated with the regulation of adipose tissue expansion. This expansion

occurs through both hypertrophy (increase in cell size) and hyperplasia (increase in cell number) (Sun et al., 2011; Jo et al., 2009)(Figure 3).

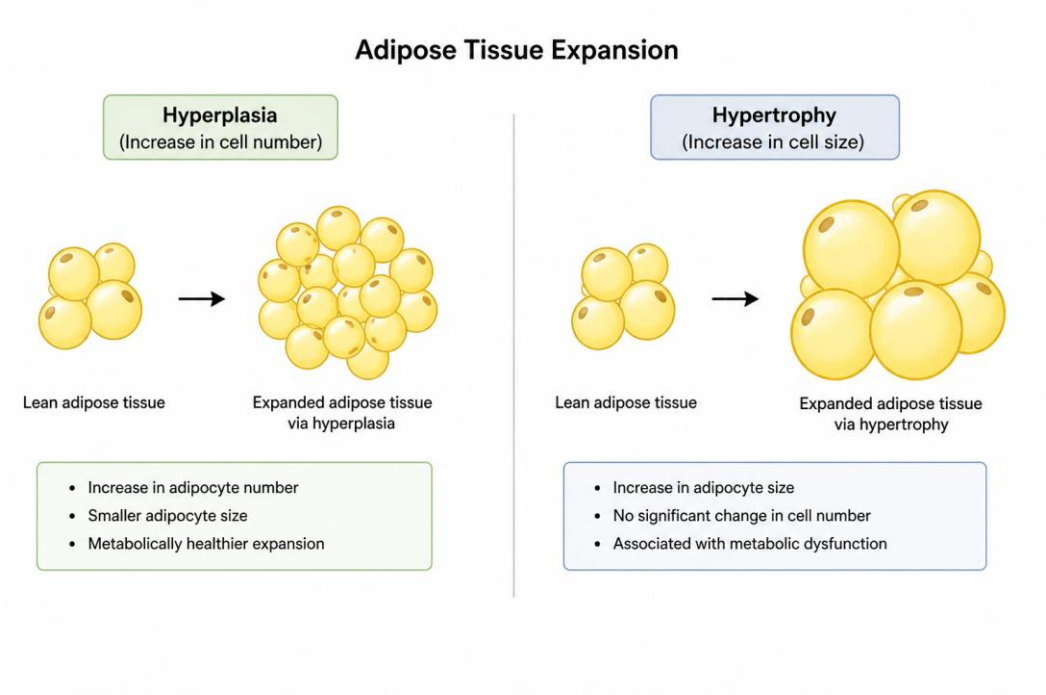

Figure 3. Adipose tissue expansion through hypertrophy and hyperplasia. *Modified from* Horwitz et al., (2023).

4.2. Adipose Tissue Biology and Adipogenesis

Adipose tissue is a highly dynamic and countable as metabolically active organ. It is not only responsible for energy storage but also plays an important role in endocrine regulation. Adipose tissue secretes a wide range of bioactive molecules, including adipokines, cytokines, and hormones, which regulate metabolism, inflammation, and insulin sensitivity (Kershaw & Flier, 2004; Rosen & Spiegelman, 2014). There are three main types of adipose tissue: white adipose tissue (WAT), brown adipose tissue (BAT), and beige adipose tissue (Figure 4). White adipose tissue is the primary site of energy storage. It stores excess energy in the form of

triglycerides and releases fatty acids when needed. Brown adipose tissue, in contrast, is specialized for energy expenditure. It generates heat through non-shivering thermogenesis, mainly via uncoupling protein 1 (UCP1) (Cannon & Nedergaard, 2004; Kajimura et al., 2015). Beige adipose tissue represents an intermediate type. It can acquire thermogenic properties under certain conditions, such as cold exposure or hormonal stimulation (Wu et al., 2012). The balance between these adipose tissue types is important for metabolic health. Excessive expansion of white adipose tissue is associated with obesity and metabolic disorders, while increased brown or beige adipose activity is linked to improved metabolic outcomes (Harms & Seale, 2013).

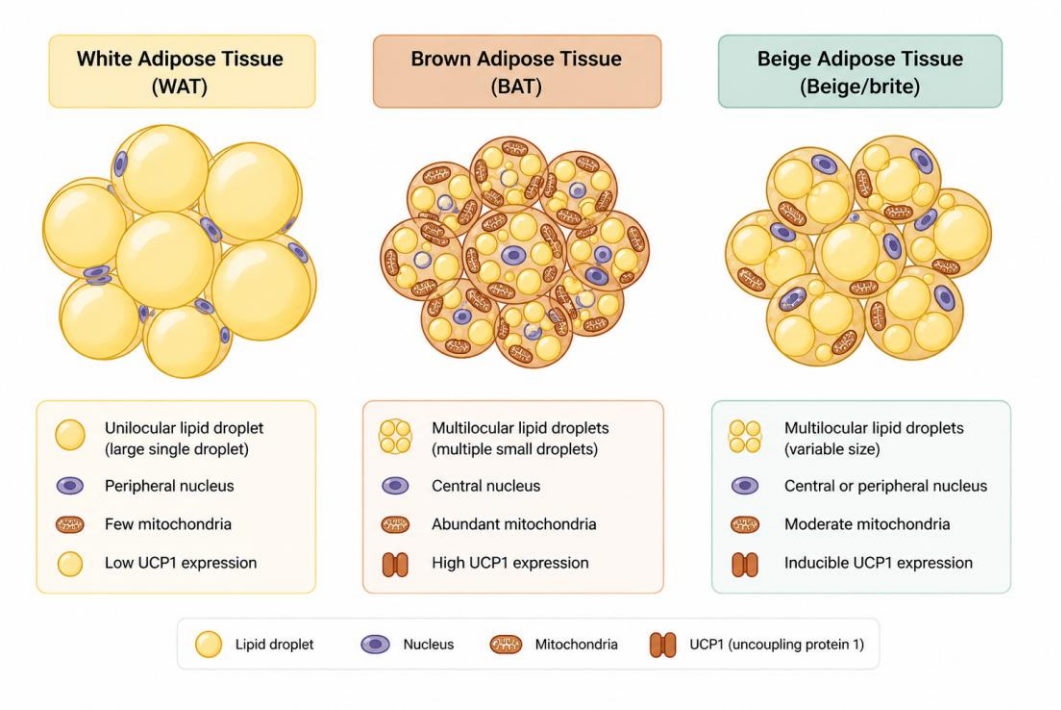

Figure 4. Comparison of white, brown, and beige adipose tissues. White adipose tissue (WAT) primarily stores energy as triglycerides, whereas brown adipose tissue (BAT) dissipates energy through UCP1-mediated thermogenesis. Beige adipose tissue represents an intermediate type with inducible thermogenic capacity. *Modified from* Hemadri et al., (2025).

Adipogenesis, the process by which new fat cells are formed, undoubtedly plays a central role in this outcome. In addition to determining the number and function of fat cells, it directly affects lipid storage capacity (Rosen & Spiegelman, 2014; Guilherme et al., 2008). Adipogenesis, the formation of fat tissue, has become a significant area of research due to its central role in both human health and animal production. This process is a complex, multistep chain of events involving the differentiation of progenitor cells into mature fat cells. It is tightly controlled over time by coordinated transcriptional and epigenetic mechanisms that regulate gene expression. Understanding these regulatory mechanisms requires a detailed examination of the biology of adipose tissue and the cellular processes underlying fat cell formation.

Adipogenesis is the process by which preadipocytes differentiate into mature adipocytes. This process occurs in a stepwise and tightly regulated manner. It includes two main stages: commitment and terminal differentiation. During the commitment stage, mesenchymal stem cells (MSCs) become preadipocytes. In the terminal differentiation stage, these cells acquire the characteristics of mature adipocytes, including lipid accumulation and expression of adipocyte-specific genes (Farmer, 2006; Cristancho & Lazar, 2011).

Adipogenesis is controlled by a complex network of transcription factors. Among these, peroxisome proliferator-activated receptor gamma (*PPARG*) is considered the master regulator of adipocyte differentiation. Activation of *PPARG* is essential for the initiation and maintenance of the adipogenic program (Tontonoz & Spiegelman, 2008). Another key group of transcription factors is the CCAAT/enhancer-binding protein (C/EBP) family. *CEBPB* and *CEBPD* are activated early during differentiation, while CEBPA acts at later stages to maintain adipocyte function (Rosen et al., 2002; Tang & Lane, 2012).

SREBF1 is another important regulator of adipogenesis. It is primarily known for its role in lipid metabolism. However, it also contributes to adipocyte differentiation by regulating lipogenic gene expression and supporting *PPARG*

activation (Horton et al., 2002; Eberlé et al., 2004). In addition to positive regulators, adipogenesis is also controlled by inhibitory factors. For example, *GATA2* acts as a negative regulator by suppressing the expression of adipogenic genes and preventing premature differentiation (Tong et al., 2000). These transcription factors function within a coordinated regulatory network. Early factors such as *CEBPB* initiate the process, followed by activation of *PPARG* and *CEBPA*, which drive terminal differentiation. This sequential activation ensures proper timing and efficiency of adipogenesis (Rosen & MacDougald, 2006; Farmer, 2006) (Figure 5).

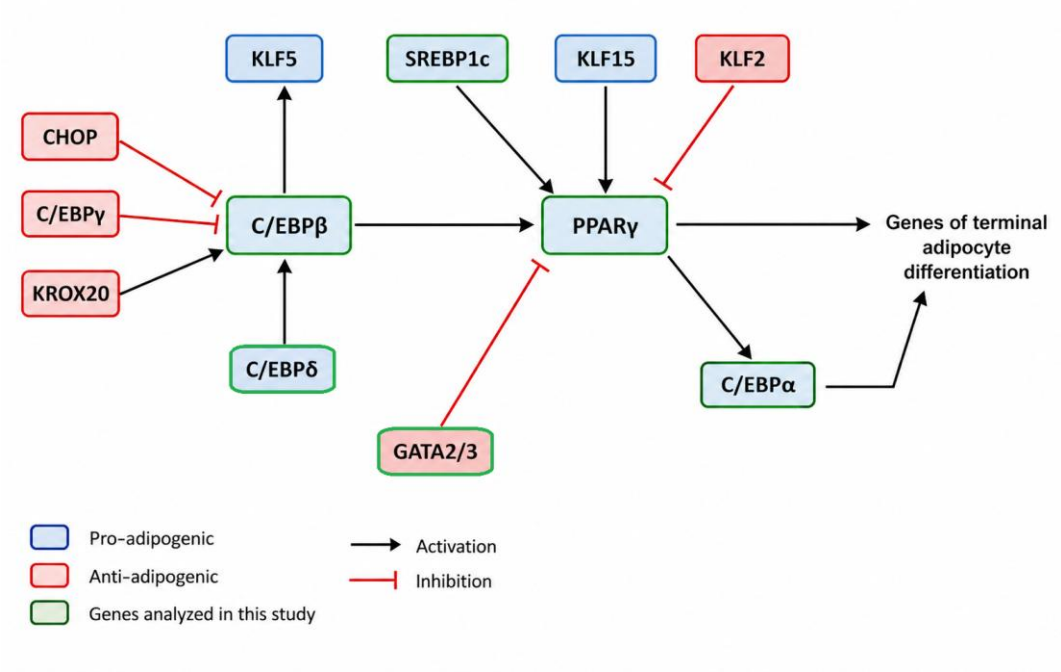

Figure 5. Transcriptional network regulating of adipogenesis. Early regulators such as C/EBPβ initiate adipogenesis, leading to activation of *PPARG*, the master regulator of adipocyte differentiation. *PPARG* subsequently induces C/*EBPα* and downstream adipocyte-specific genes. *SREBF1c* contributes to lipid metabolism, while *GATA2* acts as a negative regulator. Genes highlighted in green represent those investigated in this study. *Modified from* Zhang et al., (2020).

In addition to transcriptional regulation, adipogenesis is influenced by multiple signaling pathways. These include insulin signaling, Wnt/β-catenin signaling, and MAPK pathways. Insulin signaling promotes adipogenesis by activating downstream pathways that enhance lipid accumulation and gene expression. In contrast, Wnt signaling inhibits adipogenesis by suppressing *PPARG* and *CEBPA* expression (Christodoulides et al., 2009; Prestwich & Macdougald, 2007).

Despite extensive research, adipogenesis is not fully understood. In particular, the interaction between transcriptional regulation and chromatin structure remains unclear. Increasing evidence suggests that epigenetic mechanisms play a key role in controlling gene expression during adipocyte differentiation (Lefterova et al.,2014). Therefore, understanding adipogenesis requires not only the identification of key transcription factors but also a detailed analysis of the epigenetic mechanisms that regulate chromatin structure and gene activity. To investigate these regulatory processes, appropriate cellular models are required.

4.3. The Pig as a Model Organism

The selection of an appropriate model organism is also critical for studying complex biological processes such as adipogenesis and obesity. In recent years, the pig has emerged as a highly relevant model for human metabolic diseases. This is mainly due to its physiological and genetic similarities to humans (Swindle et al., 1994; Lunney, 2007).

Compared to commonly used rodent models, pigs show a closer resemblance to humans in several aspects. These include body size, organ development, digestive physiology, and lipid metabolism. In addition, pigs develop obesity related conditions such as insulin resistance, dyslipidemia, and cardiovascular alterations in a manner similar to humans (Swindle et al., 1994; Rosen & Spiegelman, 2014). These

similarities make pigs a valuable model for studying the mechanisms underlying metabolic disorders.

The genetic architecture of pigs also supports their use as a model organism. The pig genome has been well characterized, and many genes involved in metabolism and adipogenesis are conserved between pigs and humans. This allows the identification of candidate genes and regulatory pathways associated with obesity (Swindle et al., 1994; Groenen et al., 2012).

In addition, pigs are widely used in quantitative trait loci (QTL) studies. These studies help identify genomic regions associated with fat deposition, growth performance, and metabolic traits. Gene expression profiling and polymorphism analyses further contribute to understanding the genetic basis of adipogenesis (Andersson, 2001; Groenen et al., 2001). Beyond their role as biomedical models, pigs are also economically important livestock animals. In pig production, adipose tissue development directly affects growth performance and meat quality. Both excessive and insufficient fat deposition can have negative consequences. Excess fat reduces feed efficiency and carcass value, while insufficient fat may impair meat quality, including flavor and texture (Wood et al., 2008; Hausman et al., 2009). Adipose tissue also plays a critical role during early life stages in pigs. Adequate fat reserves are essential for thermoregulation and survival in neonatal piglets. For this reason, proper regulation of adipogenesis is important not only for production efficiency but also for animal health and welfare (Herpin et al., 2002).

Taken together, these characteristics make pigs a unique model that bridges basic research and applied animal science. They provide an opportunity to study adipogenesis in a system that is relevant to both human health and agricultural production. Given the importance of both transcriptional regulation and species-specific context, it is essential to also consider the role of epigenetic mechanisms in controlling adipogenesis.

4.4. Mesenchymal Stem Cells (MSCs) and Their Role in Adipogenesis

Mesenchymal stem cells (MSCs) are multipotent progenitor cells (Figure 6). They have the ability to differentiate into multiple cell types, including adipocytes, osteocytes, and chondrocytes. MSCs can be isolated from various tissues such as bone marrow, adipose tissue, and umbilical cord. They are widely used as an in vitro model to study differentiation processes (Pittenger et al., 1999; Dominici et al., 2006).

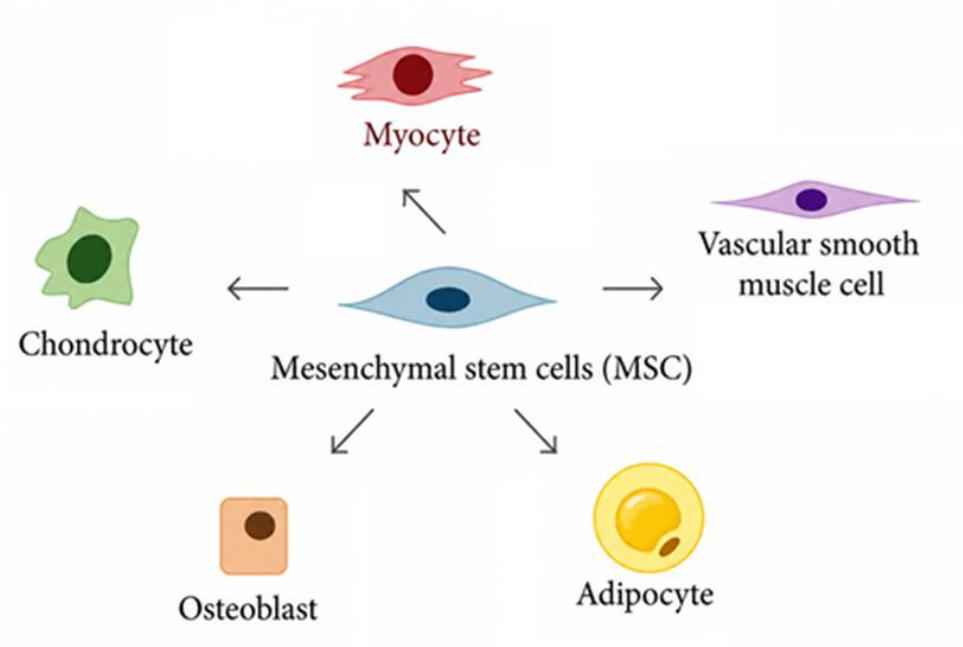

Figure 6. Differentiation potential of mesenchymal stem cells (MSCs) into multiple cell lineages, including adipocytes. *Modified from* James, (2013).

According to the criteria established by the International Society for Cell & Gene Therapy (ISCT), MSCs are characterized by plastic adherence, expression of CD105, CD73, and CD90 surface markers, lack of hematopoietic markers such as CD45 and CD34, and tri-lineage differentiation capacity (Viswanathan et al., 2019;

Choudhery et al., 2022; Warzycha et al., 2026). In the present study, the porcine adipose-derived mesenchymal stem cells (AD-MSCs) used for adipogenic differentiation experiments had been previously characterized at the Department of Molecular Biology and Mammalian Research using immunostaining and differentiation assays.

MSCs play a central role in adipogenesis. During the early stage of differentiation, MSCs commit to the adipogenic pattern and become preadipocytes. This commitment step is critical. It determines the number of adipocytes that can be formed. Once committed, these cells undergo terminal differentiation and acquire the characteristics of mature adipocytes (Rosen & MacDougald, 2006; Cristancho & Lazar, 2011).

Among different MSC sources, adipose-derived stem cells (ADSCs) are particularly important. ADSCs are easily accessible and can be obtained in large quantities. They show high proliferative capacity and strong adipogenic potential. These properties make them a valuable model for studying adipogenesis and metabolic regulation (Zuk et al., 2001; Gimble et al., 2007). ADSCs are widely used in both basic research and applied studies. They provide a reliable system for investigating gene function, signaling pathways, and differentiation mechanisms. In addition, they are highly responsive to adipogenic signals. This allows precise control of differentiation conditions in experimental settings (Bunnell et al., 2008).

The use of MSCs in adipogenesis research provides several advantages. These cells allow the study of early differentiation events that cannot be easily observed in mature adipocytes. They also provide a platform for functional studies, including gene knockout and gene regulation experiments. This is particularly important for understanding the molecular mechanisms that control adipocyte formation.

Recent studies have shown that MSC differentiation is tightly regulated at both transcriptional and epigenetic levels. Changes in chromatin structure occur during lineage commitment and influence gene expression patterns. These findings indicate that MSCs are not only a source of adipocytes but also a key system for studying regulatory mechanisms of differentiation (Siersbæk et al., 2012; Rosen &

Spiegelman, 2014). Given the importance of MSCs in adipogenesis, selecting an appropriate model system is critical. In this context, the pig has emerged as a highly relevant model organism for studying adipose tissue biology and metabolic regulation.

4.5. Epigenetic Regulation and Adipogenesis

Epigenetic mechanisms play a central role in the regulation of adipogenesis. These mechanisms control gene expression without altering the DNA sequence. They regulate chromatin structure and determine whether genes are active or silent (Kouzarides, 2007; Bird, 2007). The main epigenetic mechanisms include DNA methylation, histone modifications, and non-coding RNAs (Figure 7). Each of these mechanisms contributes to the regulation of gene expression during cell differentiation. In adipogenesis, these processes are tightly coordinated and change over time (Jaenisch & Bird, 2003; Goldberg et al., 2007).

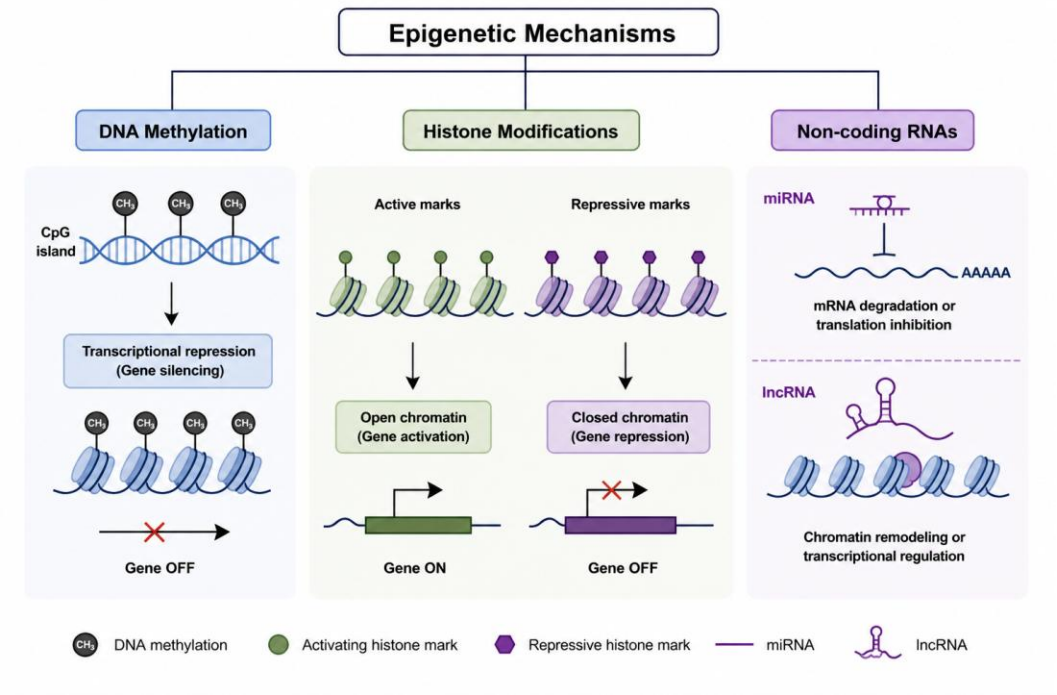

Figure 7. Overview of epigenetic mechanisms regulating gene expression. DNA methylation, histone modifications, and non-coding RNAs control chromatin structure and gene activity, leading to gene activation or repression.

DNA methylation is associated with gene repression. It occurs mainly at CpG sites and leads to reduced transcriptional activity. During adipogenesis, dynamic changes in DNA methylation patterns have been reported. These changes are important for the activation of adipogenic genes and the suppression of inhibitory pathways (Musri et al., 2010; Rönn et al., 2015).

Histone modifications are another key component of epigenetic regulation. Histone modifications, together with DNA methylation and chromatin remodeling, play a critical role in regulating gene expression by determining whether genes are in an active or silenced state (Kouzarides, 2007) (Figure 8). Histones can be modified by acetylation, methylation, phosphorylation, and other chemical changes. These modifications alter chromatin structure and influence gene accessibility. Histone acetylation is usually linked to gene activation. It promotes an open chromatin state that allows transcription factors to bind DNA (Bannister & Kouzarides, 2011).

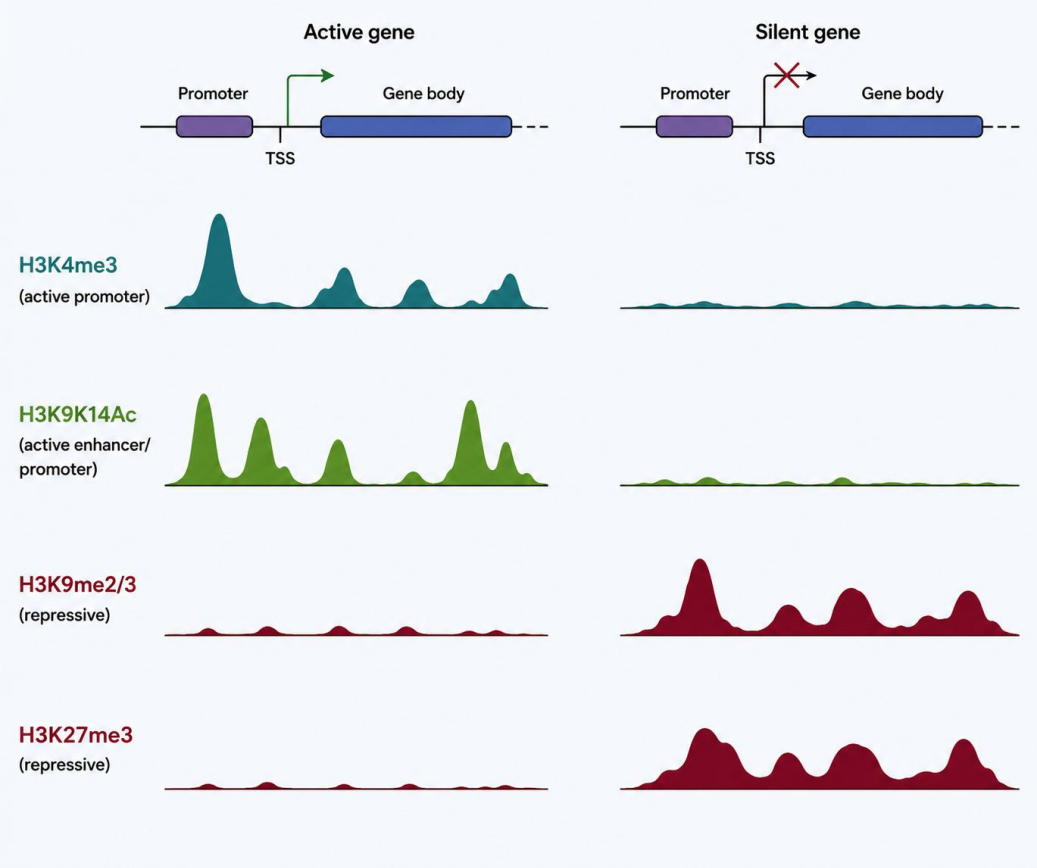

Figure 8. Distribution of histone modifications associated with active and silent gene states. Activating histone marks are enriched in transcriptionally active regions, whereas repressive marks are associated with gene silencing. *Modified from* Lim et al., (2010).

In adipogenesis, activating histone marks such as H3K9ac and H3K27ac are associated with increased expression of key genes, including *PPARG* and *CEBPA*. In opposite, repressive marks such as H3K27me3 are linked to gene silencing. These modifications change dynamically during differentiation and contribute to the timing of gene activation (Mikkelsen et al., 2010; Steger et al., 2010).

Non-coding RNAs also play an important role in adipogenesis. MicroRNAs regulate gene expression at the post-transcriptional level. Several microRNAs have

been shown to promote or inhibit adipocyte differentiation by targeting key transcription factors and signaling pathways (Zhang et al., 2013;Arner & Kulyté, 2015; Bilinska et al.,2023).

In addition to these mechanisms, chromatin organization within the nucleus also influences gene expression. The spatial arrangement of chromatin affects the accessibility of regulatory regions. Active genes are often located in open chromatin regions, while inactive genes are associated with condensed chromatin domains. This nuclear organization contributes to the regulation of adipogenic gene expression (Szczerbal et al., 2009; Cavalli & Misteli, 2013).

Recent studies suggest that epigenetic regulation is not only a supporting mechanism but also a driving force in adipogenesis. Changes in chromatin structure can precede transcriptional activation. This indicates that epigenetic modifications help define the order and timing of gene expression during differentiation (Siersbæk et al., 2011). Given the strong interaction between transcription factors and epigenetic mechanisms, understanding adipogenesis requires integrated approaches that combine gene regulation and chromatin dynamics.

4.6. CRISPR-Cas9 Gene Editing Technology

CRISPR-Cas9 is a powerful genome editing technology. It allows precise modification of DNA sequences in living cells. This system is derived from a natural defense mechanism found in bacteria (Figure 9). Bacteria use CRISPR systems to protect themselves against viral infections (Barrangou et al., 2007; Jinek et al., 2012).

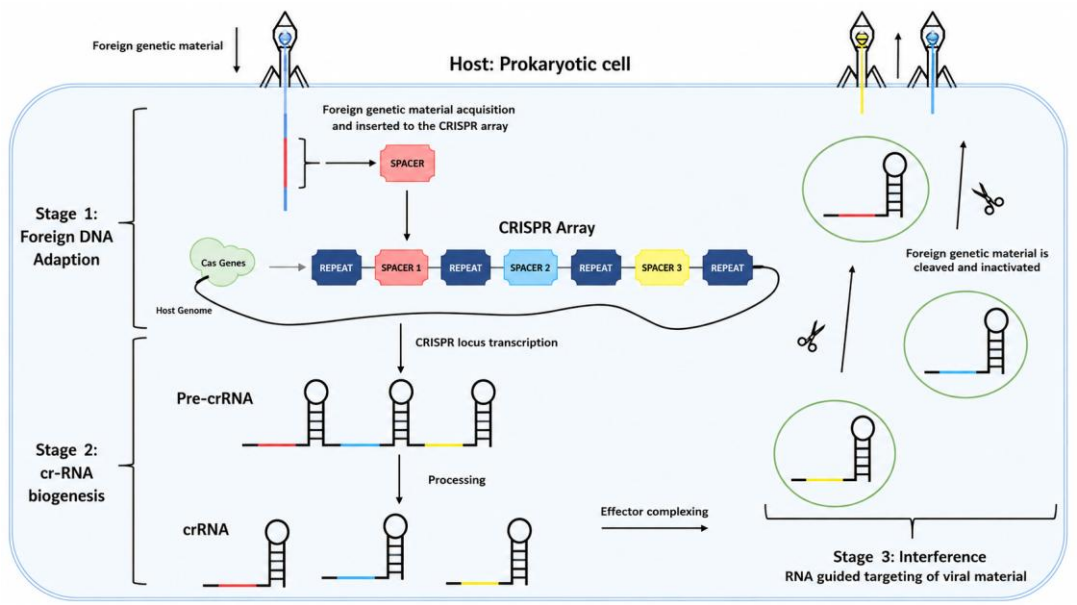

Figure 9. Schematic representation of the CRISPR-Cas immune system. The figure illustrates the stages of foreign DNA acquisition, crRNA biogenesis, and RNA-guided interference. *Previously published by the author* Rozynek et al., (2023).

The CRISPR-Cas9 system consists of two main components. The first is the Cas9 nuclease, which cuts DNA. The second is a guide RNA (gRNA), which directs Cas9 to a specific target sequence. The gRNA binds to the complementary DNA sequence, and Cas9 introduces a double-strand break at that location (Jinek et al., 2012). After DNA cleavage, the cell activates repair mechanisms. The most common repair pathway is non-homologous end joining (NHEJ). This process often introduces insertions or deletions, leading to gene disruption. Another pathway is homology-directed repair (HDR), which allows precise insertion of new genetic material when a repair template is provided (Hsu et al., 2014) (Figure 10).

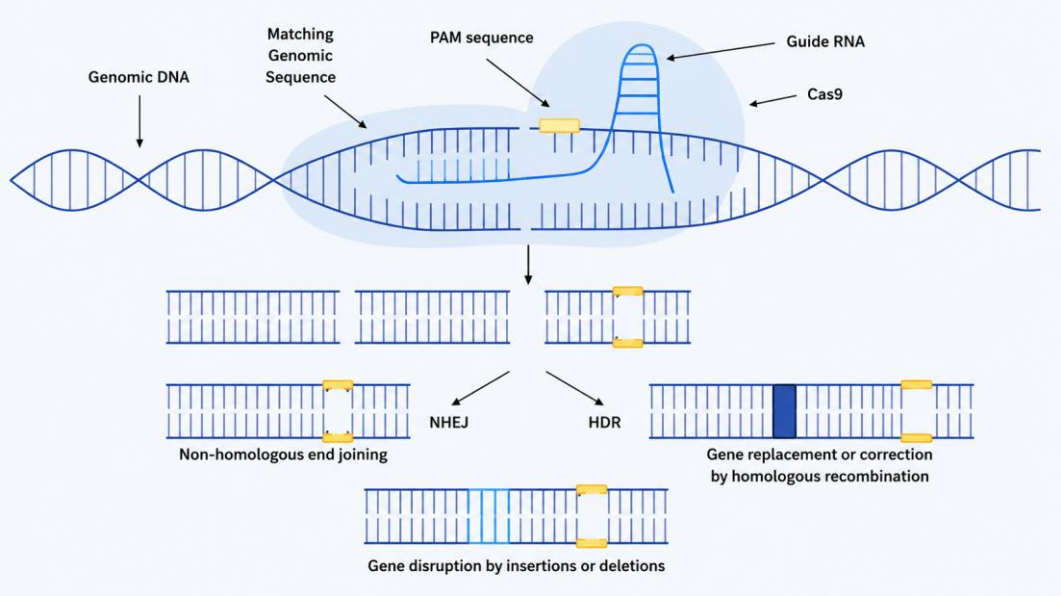

Figure 10. CRISPR/Cas9 gene editing mechanism. Cas9 guided by RNA induces a double-strand break, repaired by NHEJ or HDR pathways. *Previously published by the author* Rozynek et al., (2023).

CRISPR-Cas9 has several advantages over earlier genome editing technologies, such as zinc finger nucleases and TALENs. It is easier to design, more efficient, and highly versatile. These features have made CRISPR-Cas9 the most widely used genome editing tool in biological research (Doudna & Charpentier, 2014). This technology has been applied in many fields, including functional genomics, disease modeling, and biotechnology. In cell biology, CRISPR-Cas9 is widely used to investigate gene function by creating knockout or knock-in models. It allows researchers to study the role of specific genes in complex biological processes (Hsu et al., 2014; Doudna & Charpentier, 2014).

In the context of adipogenesis, CRISPR-Cas9 provides a powerful approach to study regulatory genes. By selectively disrupting genes, encoding crucial transcription factors it is possible to directly assess their role in adipocyte differentiation. This enables a functional understanding of transcriptional networks

and their contribution to adipose tissue development. CRISPR-Cas9 has also been successfully applied in livestock research, including pigs. Genome editing in pigs allows the study of metabolic traits and the identification of genes involved in fat deposition. These approaches are important for both biomedical research and animal production (Whitworth et al., 2014; Hai et al., 2014).

Despite its advantages, CRISPR-Cas9 has some limitations. Off-target effects may occur when the system binds to unintended genomic regions. In addition, the efficiency of HDR is relatively low compared to NHEJ. These challenges highlight the need for careful experimental design and validation (Hsu et al., 2014). Given the ability of CRISPR-Cas9 to precisely manipulate gene function, it provides an ideal tool to investigate the molecular mechanisms underlying adipogenesis and to address key unanswered questions in this field.

5. Research problem and Hypothesis

Adipogenesis is a highly regulated biological process controlled by transcriptional and epigenetic mechanisms. Previous studies have identified key transcription factors such as *PPARG*, *CEBPA*, *CEBPB*, and *SREBF1* as central regulators of adipocyte differentiation (Farmer, 2006; Rosen & Spiegelman, 2014). These factors operate in a coordinated and sequential manner to control the initiation and progression of adipogenesis.

In addition to transcriptional regulation, increasing evidence highlights the importance of epigenetic mechanisms in adipogenesis. Histone modifications, DNA methylation, and chromatin remodeling have been shown to influence gene expression and differentiation processes (Kouzarides, 2007; Mikkelsen et al., 2010). These findings suggest that adipogenesis is controlled by multiple regulatory layers.

However, despite these advances, several key questions remain unresolved. One major limitation in the current literature is the lack of a clear understanding of how transcriptional and epigenetic mechanisms are integrated during adipogenesis. In particular, it is still unclear whether changes in chromatin structure act as a cause or a consequence of transcriptional activation. This gap limits our ability to fully understand the molecular basis of adipocyte differentiation.

Another important limitation is the lack of functional studies in physiologically relevant models. While many studies have been conducted in rodent systems, fewer studies have explored these mechanisms in large animal models such as pigs. Given the physiological similarities between pigs and humans, this represents an important gap in adipogenesis research (Lunney, 2007; Groenen et al., 2012).

In addition, although key regulatory genes have been identified, their precise functional roles during different stages of adipogenesis are not fully defined. In particular, the temporal roles of early regulators such as *CEBPB* and metabolic regulators such as *SREBF1*, which has different isoforms require further investigation.

Based on the current state of knowledge and the existing research limitations, it was hypothesized that adipogenesis is regulated by interactions between transcriptional and epigenetic mechanisms, including histone modification during adipocyte differentiation. In addition, key regulatory genes, particularly *SREBF1* and *CEBPB*, were assumed to play distinct and stage-specific roles in porcine adipogenesis.

To verify this assumption, the following detailed hypotheses were formulated:

1. The *SREBF1* gene, particularly the *SREBF1c* isoform, plays a crucial regulatory role in porcine adipocyte differentiation by modulating the expression of major adipogenic genes.
2. Targeted disruption of the regulatory region of the *CEBPB* gene affects both the proliferative capacity and adipogenic differentiation potential of porcine mesenchymal stem cells.
3. Histone modifications within promoter and exon regions of adipogenic genes are dynamically associated with transcriptional activation and repression during adipocyte differentiation.
4. Changes in chromatin structure and transcription factor activity regulate the sequential activation of adipogenic genes throughout porcine adipogenesis.

6. Aim of the Study

The main objective of this study was to investigate the molecular mechanisms regulating adipogenesis in pigs, with particular emphasis on the interactions between transcriptional and epigenetic regulatory pathways controlling adipocyte differentiation.

Specific objectives:

1. To determine the functional role of the *SREBF1c* isoform in the regulation of adipogenic differentiation and the expression of key adipogenic genes in porcine mesenchymal stem cells using CRISPR/Cas9-mediated gene editing.
2. To investigate the role of the *CEBPB* gene in the proliferation and adipogenic differentiation potential of porcine mesenchymal stem cells using CRISPR/Cas9-mediated gene editing.
3. To analyze changes in selected histone modifications (H3K9ac, H4K8ac, and H4K20me3) within promoter and exon regions of four adipogenic genes *PPARG*, *GATA2*, *CEBPA*, and *CEBPB* during adipocyte differentiation.
4. To evaluate the relationship between chromatin remodeling and transcriptional activity of key adipogenic genes during porcine adipogenesis.

7. Material and Method

This doctoral study focused to the molecular mechanisms underlying porcine fat formation using mesenchymal stem cells (MSCs) through a series of interconnected experiments. All studies were conducted using a unified experimental framework, in which comparable cell culture conditions, adipogenesis protocols, and analytical approaches were applied across experiments. The core experimental procedures, including RNA isolation, gene expression analysis, and lipid accumulation assays, were consistently performed in all studies, ensuring methodological coherence. Functional analyses were carried out using CRISPR/Cas9-mediated genome editing, with different target genes selected depending on the specific objective of each study, including *SREBF1* and *CEBPB*. Additionally, epigenetic regulation was investigated through the analysis of histone modification patterns using ChIP-qPCR. Although the overall methodological approach was largely consistent, certain experiment-specific variations, particularly in gene targets and epigenetic analyses, are described in detail in the corresponding sections below.

7.1. Study Material

The study material consisted of adipose tissue-derived mesenchymal stem cells (AD-MSCs) obtained from domestic pigs of the Polish Large White (WBP) breed. Fragments of adipose tissue were collected post-mortem with the approval of the Local Ethical Committee for Animal Research at the Poznań University of Life Sciences (approval no. 57/2012). Prior to the experiments, the cells were stored in the cell bank of the Department of Genetics and Animal Breeding at the Poznań University of Life Sciences.

7.2. Cell Culture and Adipogenic Differentiation

AD-MSCs were maintained in Advanced Dulbecco's Modified Eagle Medium (Advanced DMEM; Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 5 ng/mL fibroblast growth factor-2 (FGF-2; PromoKine), 2 mM L-glutamine (Gibco), 1 mM 2-mercaptoethanol (Sigma-Aldrich), 1× antibiotic–antimycotic olution (Sigma-Aldrich), and 1× MEM non-essential amino acids (NEAA; Gibco). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 (Figure 11).

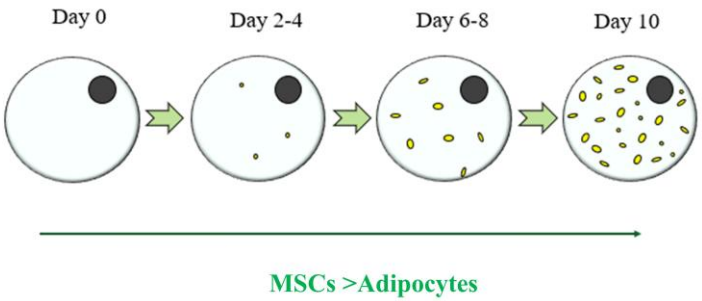

Figure 11. Schematic representation of adipogenic differentiation in mesenchymal stem cells (MSCs) over time. Cells show gradual lipid droplet accumulation from day 0 to day 10, indicating the transition from undifferentiated MSCs to mature adipocytes.

After reaching confluence, adipogenic differentiation was initiated by replacing the culture medium with differentiation medium composed of Advanced DMEM supplemented with 10% FBS, 1× antibiotic–antimycotic solution, 5 ng/mL FGF-2, 1× linoleic acid–albumin solution (Sigma-Aldrich), 1× insulin–transferrin–selenium (ITS) supplement (Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich),

100 µM indomethacin (Sigma-Aldrich), and 50 µM isobutylmethylxanthine (IBMX;Sigma-Aldrich). The differentiation process was carried out for 10 days, with the medium renewed every 24 hours.

7.3. Lipid Accumulation Analysis (BODIPY Staining)

Lipid accumulation during adipogenic differentiation was assessed using BODIPY staining (Figure 12). Cells were collected at defined time points throughout the differentiation process and fixed with 4% paraformaldehyde (PFA) for an appropriate period at room temperature. Following fixation, the cells were washed with phosphate-buffered saline (PBS) and incubated with BODIPY 493/503 dye (Invitrogen) to visualize intracellular lipid droplets.

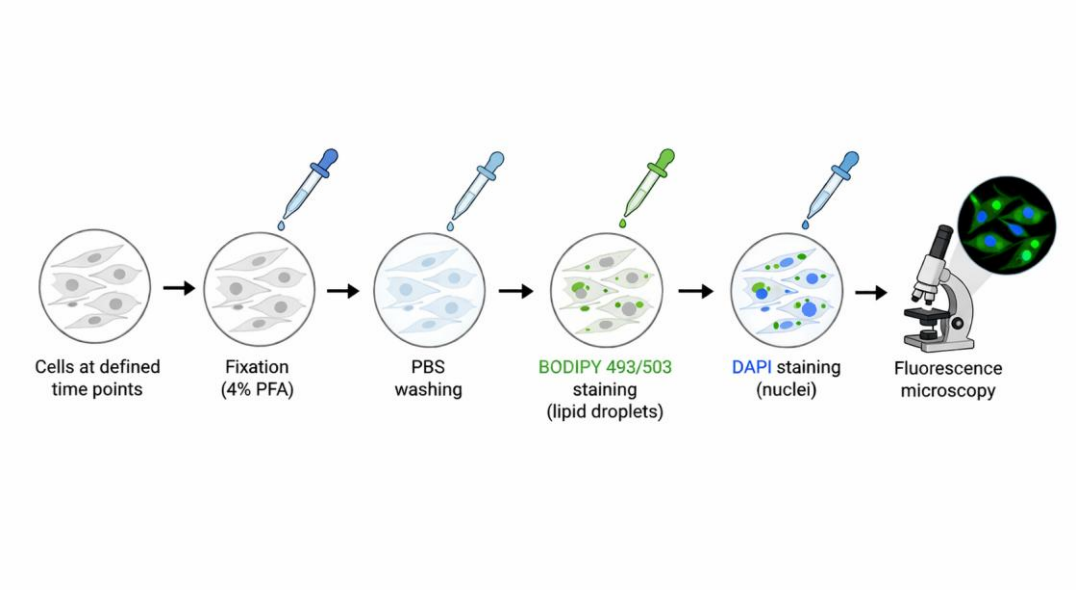

Figure 12. Workflow of lipid accumulation analysis using BODIPY staining. Cells were fixed with paraformaldehyde, washed, and stained with BODIPY 493/503 to visualize intracellular lipid droplets, followed by DAPI counterstaining for nuclei. Fluorescence microscopy was used to assess lipid accumulation during adipogenic differentiation.

After staining, cell nuclei were counterstained with DAPI, and samples were washed to remove excess dye. Fluorescence images were captured using a fluorescence microscope under standardized settings. Lipid droplet accumulation was evaluated based on fluorescence intensity and distribution within the cells. Fluorescence images were captured using a Nikon E600 Eclipse fluorescent microscope under standardized settings. This method was applied in all experiments to ensure consistent evaluation of adipogenic differentiation.

7.4. CRISPR/Cas9 Mediated Gene Editing

CRISPR/Cas9-mediated genome editing was employed to investigate the functional roles of selected genes involved in porcine adipogenesis. Guide RNAs (gRNAs) targeting specific regulatory regions of the analyzed genes were designed using the CRISPOR (http://crispor.tefor.net) online tool, taking into account on-target efficiency and potential off-target effects. The selected gRNA sequences were synthesized as complementary oligonucleotides and subsequently cloned into the pX330 expression vector containing the Cas9 nuclease and a puromycin resistance cassette, enabling both genome editing and selection of transfected cells (Figure 13).

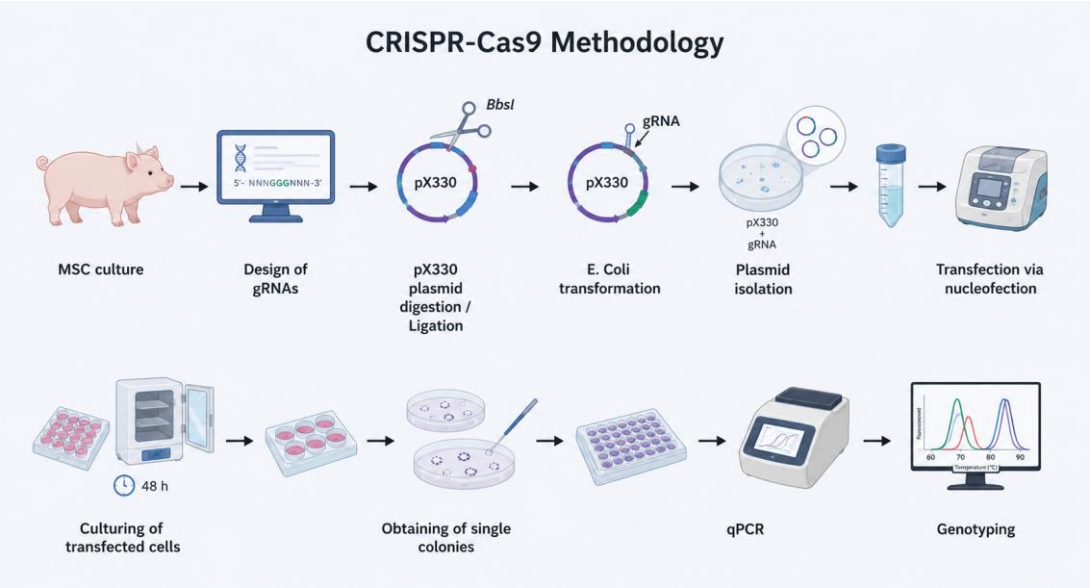

Figure 13. Workflow of CRISPR/Cas9 gene editing experiments in mesenchymal stem cells (MSCs). The process includes gRNA design, plasmid construction and its isolation, transfection of MSC via nucleofection, followed by selection of single-cell MSC colonies and downstream validation by qPCR and genotyping.

Porcine mesenchymal stem cells (MSCs) were transfected with the constructed plasmids using the 4D-Nucleofector™ system (Lonza), following the manufacturer's protocol optimized for primary cells. For each transfection, plasmids carrying two gRNAs targeting the regions flanking the sequence of interest were co-delivered to induce targeted deletions. To evaluate transfection efficiency, a control plasmid encoding green fluorescent protein (GFP) was used in parallel experiments.

Following transfection, cells were cultured under standard conditions and subjected to puromycin selection to enrich the population of successfully transfected cells. Surviving cells were then expanded and used to generate single-cell-derived colonies. Individual colonies were screened for the presence of targeted genomic modifications using PCR based genotyping, with primers designed to amplify regions spanning the expected deletion sites.

PCR products were analyzed by Sanger sequencing to confirm the presence and precise size of the introduced deletions. Sequence data were aligned to the reference genome to verify correct genome editing and to exclude unintended modifications within the analyzed regions.

The general genome editing strategy was consistent across all experiments; however, different target genes were selected depending on the specific objectives of each study, including *SREBF1* and *CEBPB*. Detailed information regarding gRNA sequences, target regions, and specific experimental parameters is provided in the corresponding publications.

7.5. PCR genotyping and Sanger Sequencing

Targeted genomic modifications introduced by CRISPR/Cas9 were verified using PCR based genotyping (Figure 14). Genomic DNA was isolated from single-cell-derived mesenchymal stem cell (MSC) colonies using a commercial DNA extraction kit, following the manufacturer's protocol. The concentration and purity of the extracted DNA were assessed spectrophotometrically.

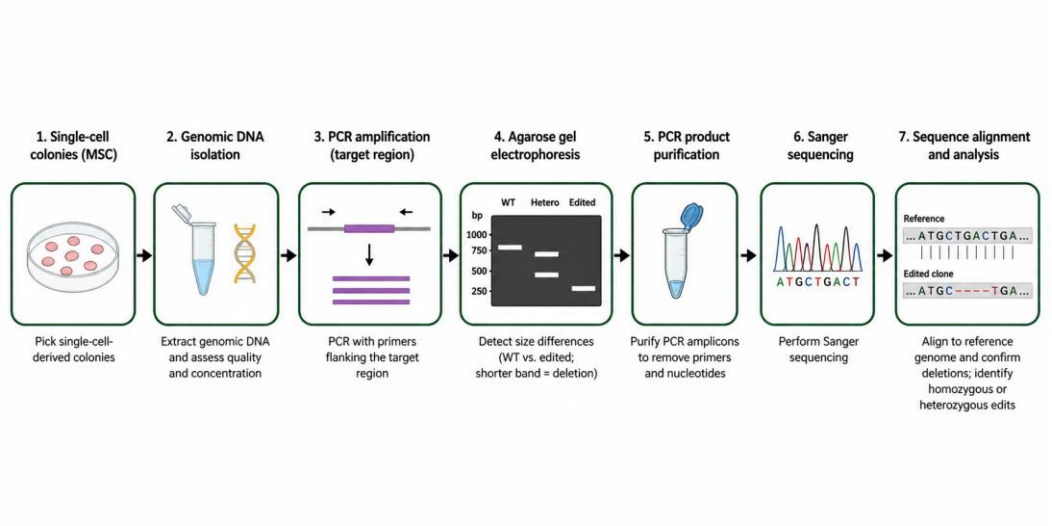

Figure 14. Workflow of CRISPR/Cas9 genotyping and validation. Genomic DNA isolated from single-cell-derived MSC colonies was amplified by PCR and analyzed by agarose gel electrophoresis to detect deletion events. PCR products were purified and subjected to Sanger sequencing, followed by alignment with the reference genome to confirm targeted modifications.

PCR primers were designed to flank the targeted regions of interest, allowing the detection of expected deletions based on fragment size differences. Amplification was performed under optimized cycling conditions, and PCR products were analyzed by agarose gel electrophoresis to distinguish between wild-type and edited alleles. The presence of shorter amplicons indicated successful deletion events within the targeted genomic regions.

To confirm the precise nature of the introduced modifications, selected PCR products were subjected to Sanger sequencing. Prior to sequencing, PCR amplicons were purified enzymatically to remove residual primers and nucleotides. Sequencing reactions were carried out using a commercial sequencing kit, and the resulting products were analyzed using capillary electrophoresis.

Obtained sequences were aligned with the reference genome to verify the exact deletion sites and to confirm the absence of unintended sequence alterations within the analyzed regions. This approach enabled accurate identification of both homozygous and heterozygous edited clones.

The described genotyping and sequencing procedures were applied consistently across all experiments.

7.6. Real-Time qPCR analysis

Total RNA was isolated from undifferentiated mesenchymal stem cells (MSCs) and MSC from the subsequent days of differentiation towards adipocytes using TriPure Isolation Reagent (Roche), following the manufacturer's instructions (Figure 15). The concentration and purity of RNA samples were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Only high-quality RNA samples were used for further analysis.

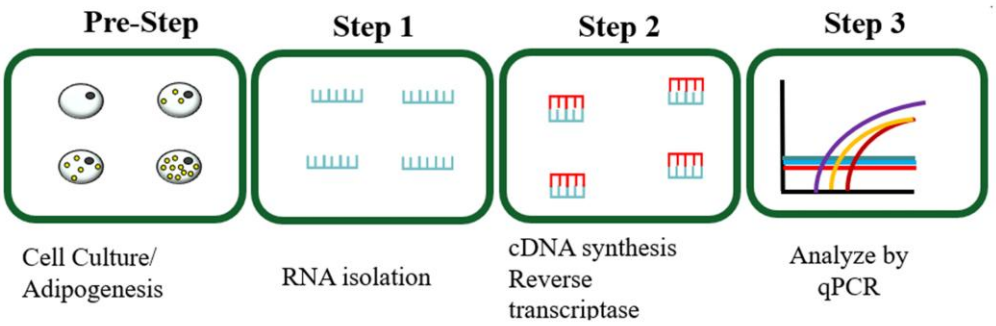

Figure 15. Workflow of gene expression analysis during adipogenesis. Cells undergoing adipogenic differentiation were subjected to RNA isolation, followed by cDNA synthesis using reverse transcription, and analyzed by quantitative PCR (qPCR) to assess gene expression levels.

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the manufacturer's protocol.

Quantitative real-time PCR (qPCR) was performed using the LightCycler 480 II system (Roche) and the LightCycler 480 SYBR Green I Master Kit (Roche). Each reaction was carried out in technical replicates under optimized cycling conditions. Primers were designed to span exon–exon junctions based on the Sscrofa11.1 reference genome to ensure amplification specificity.

Relative gene expression levels were calculated using the second derivative maximum method implemented in the LightCycler software. The *RPL27* and *PPIA* genes were used as an internal reference for normalization, and relative expression values were calculated based on the mean of biological replicates.

The selection of target genes varied depending on the experimental design and included genes associated with adipogenesis and cell proliferation. Detailed information regarding primer sequences, amplification parameters, and analyzed genes is provided in the corresponding tables of related publications. The described procedure was consistently applied across all experiments.

7.7. Chromatin Immunoprecipitation Analysis (ChIP-qPCR)

Chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) (iDeal ChIP-seq kit for Transcription Factors, Diagenode) was performed to investigate histone modification patterns associated with gene regulation during adipogenic differentiation (Figure 16). Mesenchymal stem cells (MSCs) collected at defined stages of differentiation were cross-linked with 1% formaldehyde to stabilize protein–DNA interactions. The cross-linking reaction was then quenched with glycine, and the cells were lysed to isolate chromatin.

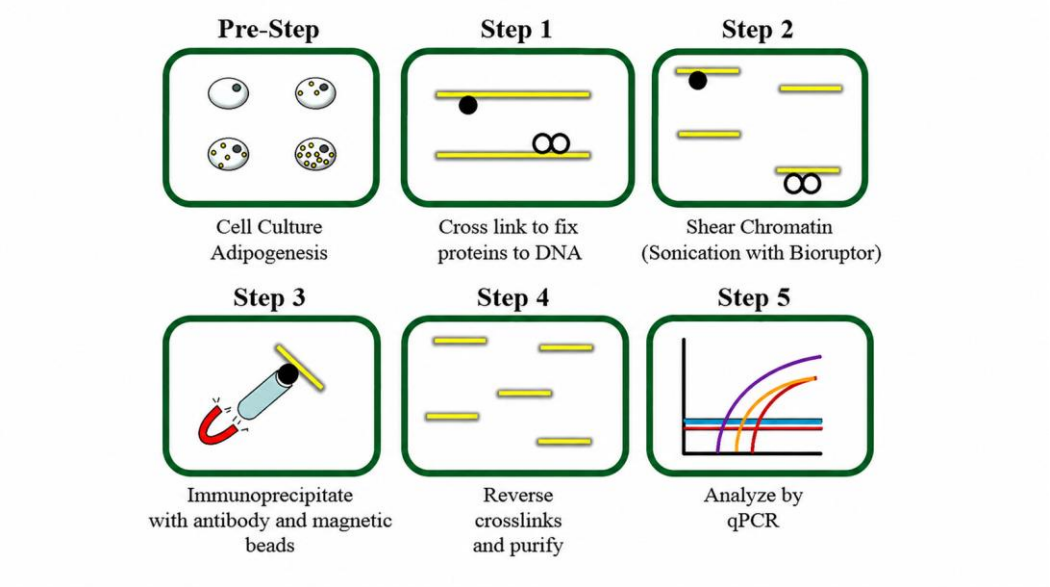

Figure 16. Workflow of chromatin immunoprecipitation (ChIP) followed by qPCR analysis during adipogenesis. Cells were crosslinked to preserve protein–DNA interactions, chromatin was sheared, and target complexes were immunoprecipitated

using specific antibodies. After reverse crosslinking and DNA purification, enrichment of target regions was analyzed by qPCR.

The isolated chromatin was fragmented by sonication (Bioruptor, Diagenode) to obtain DNA fragments suitable for immunoprecipitation. The sheared chromatin was incubated with specific antibodies recognizing the histone modifications analyzed in this study, namely H3K9ac, H4K8ac, and H4K20me3. Immunocomplexes were captured using Protein A magnetic beads, enabling selective enrichment of DNA fragments associated with the studied histone marks. Normal rabbit IgG was used as a negative control to assess non-specific binding.

Following immunoprecipitation, protein–DNA cross-links were reversed overnight at 65 °C, and the DNA was purified according to the applied protocol. The recovered DNA was subsequently used as a template for quantitative PCR analysis. Primers were designed to amplify promoter and exon regions of the analyzed genes.

ChIP-qPCR was performed using the LightCycler 480 II system (Roche, Germany) and the LightCycler 480 SYBR Green I Master Kit (Roche, Germany). Enrichment levels were calculated using the ΔCt method, normalized to input DNA, and expressed as percentage of input. Detailed information regarding the antibodies, primer sequences, and analyzed genomic regions is provided in the corresponding publication.

7.8. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 28 software (IBM Corp., Armonk) and R statistical software, depending on the experimental design. A significance level of $p < 0.05$ was considered statistically significant.

For gene expression analysis (qPCR), statistical comparisons between experimental groups and across differentiation time points were performed using

repeated measures multivariate analysis of variance (MANOVA). To assess the magnitude of observed effects, effect size measures including Pillai's trace and partial eta squared (η^2) were calculated.

Due to the relatively small sample sizes, non-parametric statistical methods were additionally applied. Differences between groups were evaluated using the Wilcoxon signed-rank test with Holm–Bonferroni correction for multiple comparisons.

For ChIP-qPCR data, statistical analysis was conducted using R software. Correlation analyses were performed using Spearman's rank correlation coefficient, and multiple testing correction was applied using the false discovery rate (FDR) method.

All qPCR measurements were performed in technical replicates, and mean values of biological replicates were used for statistical evaluation. Data are presented as mean values with appropriate measures of variability.

8. Results

8.1. First Publication

Aksoy, M. O.*, Bilinska, A.*, Stachowiak, M., Flisikowska, T., & Szczerbal, I. (2024). Deciphering the role of the *SREBF1* gene in the transcriptional regulation of Porcine adipogenesis using CRISPR/Cas9 editing. International Journal of Molecular Sciences, 25(23), 12677. https://doi.org/10.3390/ijms252312677

8.1.1. Study I – Characterization of the transcription role of *SREBF1* gene in porcine adipogenesis

The functional role of the *SREBF1* gene, with particular emphasis on the *SREBF1c* isoform, was investigated using CRISPR/Cas9-mediated genome editing in porcine adipose-derived mesenchymal stem cells (AD-MSCs) . A targeted deletion of a 567 bp fragment located within the 5′-regulatory region specific to the *SREBF1c* isoform was introduced. This approach enabled selective disruption of *SREBF1c* transcription while preserving the *SREBF1a* isoform, allowing for isoform-specific functional analysis during adipogenic differentiation.

The successful introduction of the deletion was confirmed by PCR-based genotyping and Sanger sequencing. Edited mesenchymal cells showed the expected shortened amplicon corresponding to the deleted region, confirming precise genome modification. In silico analysis of the deleted fragment revealed the presence of multiple transcription factor binding sites, including motifs associated with lipid metabolism and adipogenic regulation, indicating the functional importance of this regulatory region in controlling *SREBF1c* expression.

Expression analysis demonstrated that the *SREBF1c* isoform was completely abolished in edited cells across all analyzed time points of adipogenic differentiation.

In contrast, *SREBF1a* expression remained detectable, confirming the specificity of the deletion. In wild-type cells, *SREBF1c* expression exhibited a gradual increase during differentiation, with low levels observed at early stages (day 0–2) and a marked upregulation at later stages (day 6–10), indicating its involvement in the progression and maintenance of adipogenesis.

The expression dynamics of *SREBF1a* differed from those of *SREBF1c*. In wild-type cells, *SREBF1a* expression showed an early transient increase at day 2, followed by stabilization at later stages. In edited cells, *SREBF1a* expression was relatively higher at intermediate and late differentiation stages compared to wild-type controls. This pattern suggests a potential compensatory response to the loss of *SREBF1c*, although this compensation was not sufficient to restore normal adipogenic differentiation.

To assess the downstream effects of *SREBF1c* disruption, the expression of key adipogenic transcription factors was analyzed. A significant reduction in *PPARG* expression was observed in edited cells compared to wild-type controls, particularly at later stages of differentiation. Similarly, *FABP4* expression was markedly decreased, indicating impaired maturation of adipocytes. The expression of *CEBPA* and *CEBPD* was also reduced, suggesting disruption of the transcriptional cascade required for adipocyte differentiation. The expression of *GATA2* remain unchanged between edited and wild-type cells throughout differentiation , indicating that its regulation is not directly dependent on *SREBF1c* activity.

In contrast, *CEBPB* expression was increased in edited cells, especially at early stages of differentiation. This upregulation may reflect a compensatory mechanism or altered regulatory feedback within the adipogenic network.

The functional consequences of *SREBF1c* deletion were evaluated by monitoring lipid accumulation during adipogenic differentiation. In wild-type cells, lipid droplet formation was initiated at early differentiation stages (day 2–4) and progressively increased over time, resulting in extensive lipid accumulation at later stages (day 8–10). In contrast, edited cells lacking *SREBF1c* failed to accumulate lipid droplets at all analyzed time points. No visible morphological changes

characteristic of adipocyte differentiation were observed in these cells, indicating a complete inhibition of adipogenesis.

Additionally, expression analysis of *SREBF1* gene in adipose tissue samples revealed that *SREBF1c* expression levels were significantly higher than those of *SREBF1a* isoform in both subcutaneous and visceral fat depots. Furthermore, higher overall expression levels were observed in visceral adipose tissue compared to subcutaneous fat. These findings support the predominant role of the *SREBF1c* isoform in adipose tissue biology in pigs and are consistent with the in vitro observations.

Taken together, the results demonstrate that selective disruption of the *SREBF1c* isoform leads to impaired activation of key adipogenic genes and complete inhibition of adipocyte differentiation. The observed changes in gene expression profiles and the absence of lipid accumulation confirm that *SREBF1c* is required for proper progression of adipogenesis in porcine cells (Figure 17).

The main scientific contributions of this study include:

- for the first time, the essential role of the *SREBF1c* isoform in the regulation of porcine adipogenesis was demonstrated;
- a porcine AD-MSC model carrying a targeted deletion within the *SREBF1c* regulatory region was successfully generated using CRISPR/Cas9 genome editing, enabling functional analysis of this gene during adipogenesis;
- selective disruption of the *SREBF1c* isoform while preserving the *SREBF1a* isoform allowed the specific functions of individual *SREBF1* transcriptional variants to be investigated;
- changes in the expression profiles of major adipogenic genes (*PPARG, CEBPA, CEBPB, CEBPD, FABP4,* and *GATA2*) following *SREBF1c* disruption were characterized;

- it was demonstrated that *SREBF1c* deletion inhibits adipogenesis and prevents lipid droplet accumulation in porcine mesenchymal stem cells;
- a potential coregulatory relationship between *SREBF1c* and *CEBPB* during adipocyte differentiation was suggested;
- differences in the expression of *SREBF1a* and *SREBF1c* isoforms between subcutaneous and visceral adipose tissue in pigs were described;
- the study confirmed the usefulness of CRISPR/Cas9 technology for functional genomic studies in porcine MSCs.

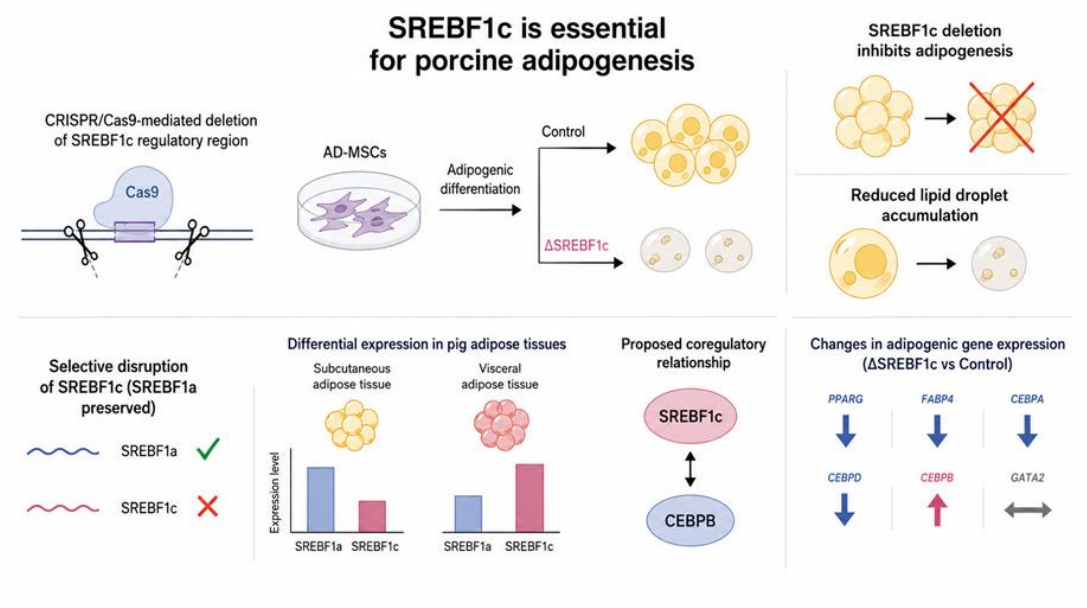

Figure 17. Schematic summary of the major findings on *SREBF1* function in pigs.

8.2. Second Publication

Aksoy, M. O., Rozynek, J., Stachowiak, M., & Szczerbal, I. (2026). The role of the *CEBPB* gene in porcine adipogenesis: a study using CRISPR/Cas9-edited mesenchymal stem cells. Journal of Applied Genetics, 67, 447-486**.** https://doi.org/10.1007/s13353-025-01030-x

8.2.1. Study II Characterization of the transcriptional role of the *CEBPB* gene in porcine adipogenesis

The functional role of the *CEBPB* gene in porcine adipogenesis was investigated using CRISPR/Cas9-mediated genome editing in adipose-derived mesenchymal stem cells (AD-MSCs) . A targeted deletion of a 575 bp fragment within the promoter region of the *CEBPB* gene was introduced to disrupt its transcriptional activity. This approach enabled the generation of both heterozygous and homozygous mutant cells, allowing for the assessment of gene dosage effects on adipogenic differentiation and cell proliferation.

Successful genome editing was confirmed by PCR-based genotyping and sequencing analysis, which verified the presence of the expected deletion within the promoter region. The introduced mutation resulted in a marked reduction of *CEBPB* transcript levels in heterozygous cells and a near-complete loss of expression in homozygous mutants, confirming effective disruption of gene transcription. The graded decrease in expression between heterozygous and homozygous cells provided a suitable model for evaluating the dose-dependent role of *CEBPB* in adipogenesis.

The effect of *CEBPB* disruption on adipogenic differentiation was assessed by analyzing the expression profiles of key transcription factors and differentiation markers. In wild-type cells, adipogenic induction resulted in a progressive increase in the expression of *PPARG*, *CEBPA*, and *FABP4*, reflecting proper activation of the

adipogenic program. In contrast, in heterozygous *CEBPB*-deficient cells exhibited significantly reduced expression of these genes across differentiation time points. Homozygous *CEBPB*-deficient cells could not be examined during differentiation because they did not proliferate and adipogenesis could not be examined.

The findings suggest that disruption of *CEBPB* alters the expression dynamics of *GATA2* during adipogenesis. Since *GATA2* is known as a negative regulator of adipocyte differentiation, its progressive downregulation in MSC wild-type-cells is consistent with normal adipogenic progression. The reduced *GATA2* expression observed in MSC with deletion cells may reflect impaired early adipogenic regulation associated with *CEBPB* deficiency.

The functional consequences of *CEBPB* disruption were further evaluated by examining lipid accumulation during adipogenic differentiation. Wild-type cells exhibited progressive lipid droplet formation, with initial accumulation observed at early stages and substantial lipid deposition at later time points. In contrast, *CEBPB* deficient cells showed a complete absence of lipid droplets. The absence of morphological features characteristic of mature adipocytes confirms that disruption of *CEBPB* leads to a functional block in adipocyte differentiation.

In addition to its role in differentiation, the effect of *CEBPB* disruption on cell proliferation was investigated. Analysis of proliferation-associated genes revealed a significant decrease in the expression of *CCND1*, *MCM2*, and *PCNA* in edited cells compared to wild-type controls. The reduction in these markers was observed in both heterozygous and homozygous mutants, with a stronger effect in the latter, further supporting a dose-dependent role of *CEBPB* in regulating cell cycle progression.

The observed decrease in proliferation markers may indicate that *CEBPB* is involved in the regulation of the mitotic clonal expansion (MCE) phase, which is a critical step in early adipogenesis. In wild type cells, this phase is characterized by a transient increase in cell proliferation following differentiation induction. In *CEBPB* deficient cells, impairment of proliferation-associated gene expression suggests disruption of this process, which may contribute to the failure of adipogenic initiation.

The simultaneous impairment of proliferation and differentiation processes indicates that *CEBPB* functions at multiple levels during adipogenesis. The disruption of early proliferation events, combined with defective activation of the transcriptional cascade, results in a comprehensive block of adipocyte formation. The persistence of these defects across differentiation stages highlights the central role of *CEBPB* in coordinating early adipogenic events (Figure 18).

Overall, the results demonstrate that *CEBPB* is essential for both the initiation of adipogenic differentiation and the regulation of cell proliferation in porcine AD-MSCs. The observed dose-dependent effects, together with the combined impairment of proliferation and differentiation, confirm that *CEBPB* acts as a critical early regulator of adipogenesis.

The main scientific contributions of this study include:

- for the first time, the functional role of *CEBPB* in porcine adipogenesis was investigated using CRISPR/Cas9-mediated genome editing;
- a porcine MSC model carrying targeted homozygous and heterozygous deletions within the *CEBPB* promoter region was successfully generated, enabling functional analysis of disrupted *CEBPB* regulation during adipogenesis;
- it was demonstrated that disruption of the *CEBPB* promoter completely inhibited adipocyte differentiation in porcine MSCs, indicating that *CEBPB* regulates not only adipogenesis but also mesenchymal stem cell proliferation;
- the obtained findings indicate that future studies focused on generating gene-edited pigs should consider target genes whose modification does not completely impair both mesenchymal stem cell proliferation and adipogenic differentiation.

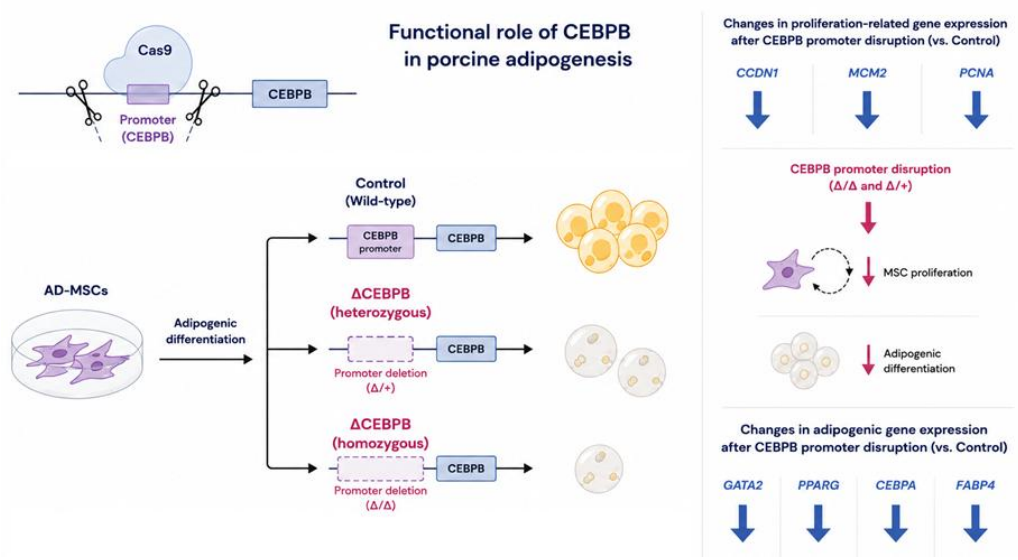

Figure 18. Schematic summary of the major findings on *CEBPB* function in pigs.

8.3. Third Publication

Aksoy, M. O., Wozniak, J., Stachowiak, M., & Szczerbal, I. (2026). Dynamic Histone Modification Patterns in Key Transcription Factor Genes During Porcine Adipogenesis. *Genes*, *17*(5), 521. https://doi.org/10.3390/genes17050521

8.3.1. Study III – Characterization of histone modifications during porcine adipogenesis

The epigenetic regulation of adipogenesis was investigated by analyzing histone modification patterns at the promoter and exon regions of key adipogenic genes during differentiation of porcine adipose-derived mesenchymal stem cells (AD-MSCs) . Chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) was used to assess the enrichment of selected histone marks associated with transcriptional activation and repression, including H3K9ac, H4K8ac, and

H4K20me3 in promoter and exon regions of *PPARG*, *GATA2*, *GP9*, *CEPBA* and *CEBPB*.

The successful occurrence of adipogenic differentiation was first confirmed by BODIPY staining. Lipid accumulation in the cells was quite low in the early stages of differentiation. Small lipid droplets began to be observed on the second and fourth days. A significant increase in lipid accumulation was seen from the sixth day onwards, and intense lipid deposition was detected in most cells on the following eighth and tenth days. These findings demonstrated that the applied culture conditions successfully induced adipocyte differentiation.

The fact that the genes *PPARG* and *GATA2*, analyzed in this study, are located on the same chromosome and play opposing transcriptional roles made this study particularly interesting. Furthermore, the histone modification profiles of both genes were found to be different. This showed that chromosomal proximity does not necessarily imply similar epigenetic regulation. Additionally, the *GP9* gene, also located on the same chromosome, was used as a control gene, revealing that a locus not associated with adipogenesis also exhibit different histone modifications.

PPARG gene expression increased gradually throughout adipogenic differentiation, as expected, and this was consistent with results obtained from our previous studies. Initially low, expression levels increased significantly, particularly after the sixth day, reaching their peak on the eighth and tenth days. Histone modification analyses showed time-dependent changes in the *PPARG* locus. H4K20me3 levels remained low in the early stages of differentiation but increased in the late stages. The H4K8ac marker increased significantly, especially in the promoter region, on the day 8 and day 10. In contrast, H3K9ac levels were high in the early stages, particularly on the second day, and decreased in subsequent days. Overall, acetylation markers were observed to play a more prominent role during *PPARG* activation.

In contrast to *PPARG*, *GATA2* gene expression was high at the beginning of differentiation and decreased as adipogenesis progressed. A significant decrease was observed on the second day. Although there was a brief increase on the fourth day,

expression levels generally remained low. When histone modifications were evaluated, H4K20me3 levels were seen to increase in the late stages. H4K8ac levels remained low in the early stages but increased on the eighth and tenth days, particularly in the exon region. The H3K9ac marker was initially found to be high in the exon region but decreased significantly from the second day onwards. These results suggested that *GATA2* suppression might be associated with a decrease in histone acetylation.

Temporal changes in histone modifications were also observed at the *GP9* locus. However, since this gene is not associated with adipogenesis, the results reflected more general changes at the chromatin level. The fact that *PPARG, GATA2,* and *GP9* genes, despite being located on the same chromosome, showed different histone modification profiles revealed that epigenetic regulation is gene-specific.

CEBPA gene expression gradually increased during adipogenesis, reaching its highest level on the sixth day. Although expression decreased in subsequent days, it remained above the baseline level. When histone modifications were examined, the H4K20me3 marker in the promoter region was found to be significantly increased, especially on the fourth day. H4K8ac levels were found to be high in the exon region on the second day and then decreased. H3K9ac was initially found to be at a high level but decreased significantly after the second day. This suggests that early histone acetylation may be important for *CEBPA* activation.

Comparative analysis of histone modification patterns across different genes revealed a coordinated epigenetic program underlying adipogenic differentiation. Early-stage regulators, such as *CEBPB*, exhibited early enrichment of activating marks, while master regulators and late-stage genes, including *PPARG* and *CEBPA*, showed progressive increases in acetylation at later stages. This temporal coordination of histone modifications reflects the sequential activation of the adipogenic transcriptional network.

CEBPB expression, showed a transient and dynamic profile. A significant increase in expression was observed on the fourth day, followed by a decrease in levels. H4K20me3 levels remained generally stable, but an increase was observed in

the promoter region on the tenth day. The H4K8ac marker remained at low levels and showed small fluctuations. The H3K9ac marker increased significantly, especially in the exon region, on the second day and then decreased in subsequent periods.

Overall, it was observed that active histone acetylation marks, such as H3K9ac and H4K8ac, often appeared before the increase in gene expression. This suggested that histone acetylation may be a mechanism that prepares chromatin for transcription. The fact that acetylation levels in promoter regions were in most cases higher than in exon regions indicated that epigenetic regulation occurs particularly at the promoter level. In contrast, the H4K20me3 mark did not show a clear association with gene expression.

Although some strong trends were observed in correlation analyses, statistically significant associations were not obtained after multiple testing correction. However, the stronger correlations found in exon regions compared to promoter regions suggested that histone modifications may play a role in chromatin organization throughout the gene body.

In conclusion, this study revealed that histone modifications during porcine adipogenesis vary in a gene and region-specific manner. Active histone marks were particularly prominent during transcriptional activation of genes associated with adipogenesis. In contrast, the repressive histone marker H4K20me3 was found to have a weaker association with transcription. These findings indicate that chromatin rearrangement during porcine adipogenesis is a dynamic and multilayered process (Figure 19).

The main scientific contributions of this study include:

- dynamic changes in histone modification patterns were identified during porcine adipogenic differentiation, indicating stage-dependent chromatin remodeling throughout adipogenesis;
- activating histone marks, particularly H3K9ac and H4K8ac, showed gene- and region-specific enrichment patterns associated with transcriptional activation of key adipogenic regulators;

- *PPARG* locus demonstrated increased histone acetylation during intermediate and late stages of differentiation, corresponding to elevated gene expression levels;
- *CEBPB* exhibited early enrichment of activating histone marks, supporting its role as an early regulator of the adipogenic transcriptional cascade;
- GATA2 showed a decrease in H3K9ac enrichment, one of the activating histone markers during differentiation, while it showed an increase in H4K20me3, which is repressive, consistent with transcriptional repression of this inhibitor of lipid formation.
- despite their close chromosomal localization, PPARG and GATA2 displayed distinct histone modification profiles, suggesting that local chromatin context may contribute to gene-specific epigenetic regulation;
- the obtained findings demonstrate that adipogenic differentiation in porcine mesenchymal stem cells is accompanied by coordinated and dynamic chromatin remodeling processes.

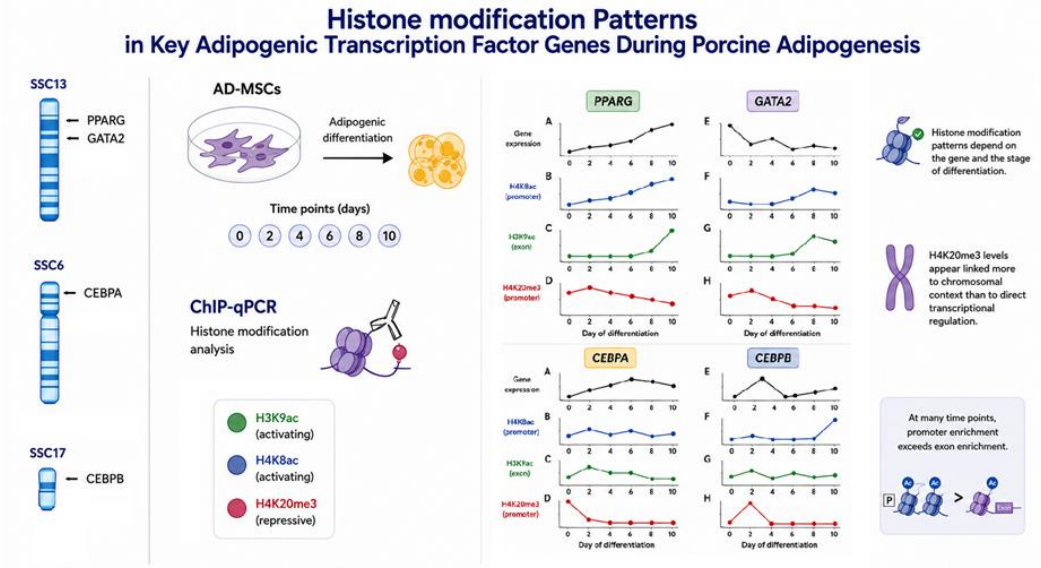

Figure 19. Schematic summary of histone modifications.

9. List of analyses performed by the doctoral candidate and by other contributors

The majority of experimental work included in this doctoral thesis was performed by the doctoral candidate. This comprises cell culture experiments, CRISPR/Cas9 genome editing, RNA isolation, gene expression analysis (RT-qPCR), and chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR).

The doctoral candidate was also actively involved in experimental design, data analysis, and interpretation of the results across all studies. Additionally, the doctoral candidate contributed to the preparation of original manuscript drafts and the revision process.

Certain experimental procedures and analyses were performed in collaboration with co-authors, particularly within the scope of methodology development and data validation. Supervision, project administration, and funding acquisition were conducted by the principal investigator.

10. Discussion

10.1. Transcriptional Regulation of Adipogenesis

Adipogenesis is a complex and tightly regulated process governed by a hierarchical network of transcription factors that coordinate both the initiation and progression of cellular differentiation. Among these, members of the CCAAT/enhancer-binding protein (C/EBP) family and peroxisome proliferator-activated receptor gamma (PPARG) are widely recognized as central regulators of adipocyte formation. Early adipogenic events are initiated by transcription factors such as *CEBPB*, which subsequently activate downstream regulators including *PPARG* and *CEBPA*, ultimately driving terminal differentiation (Farmer, 2006; Rosen & MacDougald, 2006; Aksoy et al., 2024).

The results obtained in the present study for the pig are consistent with this established model of transcriptional regulation of adipogenesis. This study provide functional evidence for the critical role of *CEBPB* in porcine adipogenesis. The CRISPR/Cas9-mediated disruption of *CEBPB* resulted in a significant downregulation of *PPARG*, *CEBPA*, and *FABP4* expression, accompanied by a complete inhibition of lipid accumulation. These findings are in agreement with previous studies demonstrating that *CEBPB* is essential for the activation of the adipogenic transcriptional cascade (Tang et al., 2003; Aksoy et al., 2024). In particular, the absence of early *CEBPB* activation observed in this study supports the notion that adipogenic commitment is highly dependent on early transcriptional events, and that disruption at this stage leads to irreversible impairment of differentiation.

In addition to its role in transcriptional activation, *CEBPB* has been shown to regulate mitotic clonal expansion (MCE), a critical early phase of adipogenesis during which preadipocytes undergo several rounds of cell division before terminal differentiation (Tang & Lane, 2012). Although MCE has been mainly characterized

in rodent adipogenesis models, early proliferative events are also relevant in porcine adipogenic systems. Studies using porcine preadipocytes have shown that changes in cell proliferation are associated with altered adipogenic differentiation (Zhao et al., 2021; Wojciechowicz et al., 2023). The observed decrease expression in proliferation markers such as *CCND1*, *MCM2*, and *PCNA* in CEBPB-deficient cells is consistent with this function and suggests that *CEBPB* integrates signals controlling both proliferation and differentiation. This dual role highlights the importance of *CEBPB* as a key coordinator of early adipogenic processes and indicates that failure of MCE may contribute to the observed block in adipogenesis. In addition, although proliferation marker expression supported altered proliferative activity in CEBPB-deficient cells, future studies could further validate these findings using additional proliferation assays such as EdU incorporation, BrdU labeling, Ki-67 staining, or cell cycle analysis (Chehrehasa et al., 2009; Sun & Kaufman, 2018).

While *CEBPB* acts as an early regulator, the results of this study demonstrate that *SREBF1* plays a distinct but equally critical role at later stages of adipogenesis. *SREBF1*, particularly its *SREBF1c* isoform, has been extensively characterized as a regulator of lipid biosynthesis and metabolic homeostasis (Horton et al., 2002). However, increasing evidence suggests that *SREBF1* is also involved in adipocyte differentiation through the regulation of lipogenic gene expression and interaction with other transcription factors (Eberlé et al., 2004).

The findings presented here provide direct functional evidence supporting this extended role of *SREBF1*. Selective disruption of the *SREBF1c* isoform resulted in a complete inhibition of adipocyte differentiation, as indicated by the absence of lipid droplet formation and reduced expression of key adipogenic markers, including *PPARG* and *CEBPA*. These observations are consistent with previous reports demonstrating that *SREBF1* activity is required for the full activation of adipogenic genes and lipid accumulation (Kim & Spiegelman, 1996; Shimano & Sato, 2017). Importantly, the temporal expression pattern observed in this study, characterized by increased *SREBF1c* expression at later stages of differentiation, further supports its role in maintaining and amplifying the adipogenic program.

A notable observation from this study is the upregulation of *CEBPB* expression in SREBF1 deficient cells, suggesting the presence of compensatory mechanisms within the transcriptional network. Similar compensatory interactions between transcription factors have been described in adipogenesis, where disruption of one regulatory component can lead to the activation of alternative pathways (Wang et al., 2013; Rosen & Spiegelman, 2014; Lefterova et al.,2014). However, despite this compensatory increase in *CEBPB* expression, adipogenesis was not restored, indicating that the functions of *SREBF1* and *CEBPB* are not redundant but rather temporally and functionally distinct.

Taken together, the results of this study support a model in which adipogenesis is regulated by a sequential transcriptional hierarchy involving early and late regulators. *CEBPB* functions as an early transcription factor required for the initiation of adipogenic commitment and proliferation, whereas *SREBF1* contributes to the progression and completion of differentiation by regulating lipid metabolism and gene expression. Disruption of either component leads to failure of adipocyte formation, highlighting the necessity of coordinated transcriptional regulation.

These findings not only confirm the established roles of *CEBPB* and *SREBF1* in adipogenesis but also provide new insights into their functional interplay in porcine cells. The integration of these results into the broader context of adipogenic regulation underscores the importance of temporal coordination between transcription factors and suggests that both early and late regulatory mechanisms are essential for successful adipocyte differentiation.

10.2. Epigenetic Regulation of Adipogenesis

Adipogenesis is not controlled only by transcription factors. Epigenetic mechanisms also play a central role. These mechanisms regulate chromatin structure and determine whether genes can be activated or silenced. Among them, histone

modifications are particularly important for controlling gene expression during differentiation (Kouzarides, 2007).

Histone acetylation is generally associated with gene activation. It leads to a more open chromatin structure, allowing transcription factors to access DNA. In contrast, some histone methylation marks are linked to transcriptional repression, although their effects depend on the specific residue involved (Bannister & Kouzarides, 2011). In adipogenesis, dynamic changes in histone modifications have been reported to accompany the activation of key regulatory genes such as *PPARG* and *CEBPA* (Musri et al., 2010).

The results obtained in this study support this model. Increased enrichment of activating histone marks, specifically H3K9ac and H4K8ac, was observed at the promoter regions of key adipogenic genes. This increase correlated with elevated gene expression levels during differentiation. In particular, *PPARG* showed a progressive increase in histone acetylation over time. This pattern was consistent with its role as a master regulator that is activated during the later stages of adipogenesis.

A similar trend was observed for *CEBPA*. The enrichment of activating histone marks increased gradually during differentiation. This suggests that chromatin remodeling at this locus is a stepwise process. These findings are in line with previous studies showing that activation of *CEBPA* is associated with increased histone acetylation and chromatin accessibility (Steger et al., 2010).

In contrast, the *CEBPB* gene showed a different pattern. High levels of histone acetylation were detected at early stages of differentiation. This supports the idea that *CEBPB* is an early-response gene. Its activation occurs rapidly after induction and precedes the activation of downstream regulators. Similar early epigenetic activation of *CEBPB* has been described in previous studies, where chromatin remodeling was shown to be required for its rapid transcriptional induction (Guo et al., 2015).

The *GATA2* gene displayed an opposite trend. A decrease in histone acetylation was observed during differentiation. This reduction was associated with decreased gene expression. *GATA2* is known to act as a negative regulator of

adipogenesis. Its repression is required for differentiation to proceed (Tong et al., 2000). The results of this study are consistent with this role and indicate that epigenetic silencing contributes to the downregulation of anti-adipogenic factors.

An additional important observation was that *PPARG* and *GATA2* displayed distinct histone modification profiles despite their close chromosomal localization. This suggests that epigenetic regulation during adipogenesis is highly locus-specific rather than coordinated across large chromosomal regions. Local chromatin accessibility, transcription factor recruitment, and regulatory element activity may therefore play more important roles in determining gene activity than genomic proximity alone (Bonev & Cavalli, 2016; Schoenfelder & Fraser, 2019).

In contrast to histone acetylation marks, the relationship between H4K20me3 enrichment and transcriptional activity appeared less direct. Although dynamic changes in H4K20me3 levels were detected during differentiation, these changes did not consistently correlate with gene expression patterns. This may indicate that H4K20me3 is involved less in rapid transcriptional regulation and more in higher-order chromatin organization, chromatin compaction, or stabilization of cellular identity during differentiation. The relatively stable nature of this modification may also explain why its enrichment patterns were less tightly associated with temporally dynamic adipogenic gene activation (Beck et al., 2012).

The observation that increased histone acetylation frequently preceded maximal gene expression suggests that chromatin remodeling may function as a priming mechanism during adipogenesis. In this context, early histone acetylation could establish a permissive chromatin environment before full transcriptional activation occurs. Such chromatin priming may facilitate recruitment of adipogenic transcription factors and transcriptional machinery during lineage commitment and differentiation progression (Siersbæk et al., 2017; Ghaben & Scherer, 2019).

Another important observation was the stronger enrichment of histone acetylation marks within promoter regions compared to exon regions. This suggests that epigenetic regulation during adipogenesis is concentrated primarily at transcriptional control regions. Increased promoter acetylation may facilitate

transcription factor binding and recruitment of transcriptional machinery, thereby promoting gene activation. In contrast, histone modifications within exon regions may be more closely associated with transcriptional stability and maintenance of chromatin organization across the gene body (Bannister & Kouzarides, 2011; Kundaje et al., 2015).

Taken together, these findings indicate that histone acetylation plays a central role in regulating adipogenic gene expression. Activation of adipogenic genes is associated with increased acetylation, while repression of inhibitory genes is linked to decreased acetylation. This coordinated pattern ensures proper timing of gene activation during differentiation.

Importantly, the temporal aspect of these changes is critical. Early regulators such as *CEBPB* show early epigenetic activation, while master regulators such as *PPARG* and *CEBPA* are activated later. This sequential pattern reflects the hierarchical nature of adipogenesis and suggests that epigenetic mechanisms help define the order of gene activation.

The sequential activation pattern observed in this study likely reflects progressive changes in chromatin accessibility during adipogenesis. Early regulators such as *CEBPB* may initiate chromatin remodeling events that subsequently permit activation of downstream adipogenic regulators, including *PPARG* and *CEBPA*. This hierarchical organization may help ensure that adipocyte differentiation proceeds in a coordinated and irreversible manner. Such temporal coordination between chromatin remodeling and transcription factor activity is considered a fundamental feature of lineage commitment and cellular differentiation (Dixon et al., 2016; Madsen et al., 2018).

Overall, the results demonstrate that adipogenesis is controlled by dynamic and gene-specific changes in histone modifications. These changes are closely linked to transcriptional activity and are essential for the progression of differentiation. The findings are consistent with previous studies and provide additional evidence that chromatin remodeling is a key regulatory layer in adipogenesis.

10.3. Integrated Transcriptional and Epigenetic Control of Adipogenesis

Both transcription factors and epigenetic mechanisms regulate adipogenesis. These two layers do not act independently. Instead, they work together to control the timing and progression of gene activation. The results obtained in this study clearly support this integrated model.

Transcription factors such as *CEBPB*, *SREBF1*, and *PPARG* define the regulatory hierarchy of adipogenesis. However, their activity depends on chromatin accessibility. Without appropriate chromatin remodeling, transcription factors cannot efficiently bind to their target genes. This relationship between transcriptional and epigenetic regulation has been widely described in previous studies (Rosen & Spiegelman, 2014; Siersbæk et al., 2012; Shao et al.,2018).

The results of this thesis show that early transcriptional activation is closely linked to early epigenetic changes. *CEBPB*, identified as an early regulator, exhibited both early gene expression and early enrichment of activating histone marks. This suggests that chromatin remodeling is required for its rapid activation. Similar observations have been reported, where early adipogenic transcription factors bind to regions that become accessible prior to full gene activation (Siersbæk et al., 2011; Lee et al., 2019; Madesn et al., 2020).

At later stages of differentiation, genes such as *PPARG* and *CEBPA* showed increased histone acetylation and expression. This indicates that epigenetic modifications are not only required for initiation but also for maintaining transcriptional activity. These findings are consistent with studies demonstrating that sustained chromatin accessibility is necessary for stable adipocyte differentiation (Lefterova et al., 2008: Ge., 2012; Stachecka et al.,2021).

The role of *SREBF1* further supports the integration of transcriptional and epigenetic regulation. Although *SREBF1* is primarily known as a transcription factor regulating lipid metabolism, its activity is also dependent on chromatin context. The complete inhibition of adipogenesis observed after *SREBF1c* disruption suggests that

transcriptional regulation alone is not sufficient. Proper chromatin activation is also required for downstream gene expression. Previous studies have shown that SREBP proteins can interact with co-activators and chromatin-modifying enzymes, linking transcriptional regulation with epigenetic control (Näär et al., 2002; Matsumura et al., 2022).

An important observation from this study is the sequential nature of these regulatory events. Early regulators such as *CEBPB* are activated first, followed by intermediate and late regulators such as *CEBPA* and *PPARG*. This sequence is mirrored by changes in histone acetylation. Early genes show early acetylation, while late genes show delayed activation. This coordinated pattern suggests that epigenetic mechanisms help define the temporal order of transcriptional events.

The repression of inhibitory genes also follows this integrated model. The decrease in histone acetylation at the *GATA2* promoter was associated with reduced gene expression. This indicates that epigenetic silencing is required to remove barriers to adipogenesis. Similar mechanisms have been described, where repression of anti-adipogenic factors is necessary for differentiation to proceed (Tong et al., 2000; Moreno-Navarrete et al., 2017).

Taken together, these findings support a model in which transcription factors and epigenetic modifications function as a coordinated regulatory system. Transcription factors define which genes should be activated, while epigenetic mechanisms determine when and how efficiently this activation occurs. Disruption of either layer leads to failure of adipogenesis.

Overall, the integration of transcriptional and epigenetic regulation provides a more comprehensive understanding of adipogenesis. The results presented in this thesis demonstrate that successful differentiation requires both correct transcription factor activity and proper chromatin remodeling. This combined regulation ensures precise control of gene expression and highlights the complexity of adipogenic processes.

10.4. Biological and Physiological Implications of Adipogenic Regulation

Adipogenesis is not only a cellular process. It also has important biological and physiological consequences. Proper regulation of adipocyte differentiation is essential for maintaining metabolic balance. Disruption of this process can lead to metabolic disorders, including obesity and insulin resistance (Rosen & Spiegelman, 2014; Cohen & Spiegelman, 2016; Longo et al., 2019).

The results of this study provide insight into how adipogenesis is controlled at the molecular level in porcine cells. These findings are relevant not only for basic biology but also for animal production and human health. In pigs, adipose tissue plays a key role in determining meat quality. The amount and distribution of fat influence both flavor and texture. Therefore, understanding the regulation of adipogenesis has direct applications in livestock production (Hausman et al., 2009; Hocquette, 2010).

The identification of *SREBF1* and *CEBPB* as critical regulators highlights their importance in controlling fat deposition. Disruption of either gene resulted in a complete inhibition of adipocyte differentiation. This suggests that these factors are essential for normal adipose tissue development. In practical terms, modulation of their activity could influence fat accumulation in animals. Similar regulatory mechanisms have been described in other species, indicating that these pathways are conserved (Farmer, 2006; Cristancho & Lazar, 2011; Abebe, 2024).

The role of *CEBPB* in early adipogenesis is particularly relevant. Its involvement in both proliferation and differentiation suggests that it controls the initial expansion of adipocyte precursors. This phase is important for determining the final number of adipocytes. An increased number of adipocytes is often associated with obesity, whereas impaired adipogenesis can lead to ectopic fat deposition in non-adipose tissues (Sun et al., 2011). Therefore, regulation of early adipogenic events has significant physiological implications.

SREBF1, on the other hand, appears to be more important for the later stages of adipogenesis. Its role in lipid metabolism links adipocyte differentiation with lipid

storage capacity. Proper activation of *SREBF1* ensures that differentiating cells can accumulate lipids efficiently. This function is essential for the formation of mature adipocytes and for maintaining energy homeostasis (Shimano, 2009; Fayyad et al., 2019; Abebe et al., 2024; Li et al., 2026).

The epigenetic findings of this study also have important implications. The observed changes in histone acetylation indicate that chromatin remodeling is required for proper gene activation. This suggests that environmental factors could influence adipogenesis through epigenetic mechanisms. Diet, stress, and metabolic status are known to affect chromatin structure and gene expression (Siersbæk et al., 2012; Kim et al., 2019; Ghaben & Scherer, 2019). Therefore, adipogenesis may be regulated not only by genetic factors but also by external conditions.

Another important aspect is the potential relevance of these findings to human health. Although this study was performed in porcine cells, pigs are considered a valuable model for human metabolism due to physiological similarities. The regulatory pathways identified in this study are likely conserved across species. This increases the relevance of the findings for understanding human adipose tissue biology (Hausman et al., 2009; Kleinert et al., 2018).

Dysregulation of adipogenesis is a key feature of metabolic diseases. Excessive adipogenesis can contribute to obesity, while insufficient adipogenesis can lead to lipodystrophy. Both conditions are associated with metabolic complications, including insulin resistance and type 2 diabetes (Rosen & Spiegelman, 2014). Understanding the molecular mechanisms controlling adipogenesis may help identify new therapeutic targets.

The integration of transcriptional and epigenetic regulation further emphasizes the complexity of adipogenesis. It suggests that effective interventions may need to target multiple regulatory levels. For example, modulating transcription factor activity alone may not be sufficient if chromatin accessibility is not properly regulated. This highlights the need for a more comprehensive approach to studying and manipulating adipogenic processes.

Overall, the findings of this study contribute to a better understanding of adipose tissue development and function. They provide a link between molecular regulation and physiological outcomes. This knowledge can be applied in both agricultural and biomedical contexts. It also underscores the importance of precise regulation of adipogenesis for maintaining metabolic health.

10.5. Limitations and Future Perspectives

This study provided several novel and valuable findings, while also revealing certain limitations that may guide future research directions.

First, most functional conclusions are based on mRNA expression data. The transcript changes were consistent with the observed cellular phenotypes. However, protein level validation was not included. This is important because mRNA levels do not always directly predict protein abundance or protein activity (Vogel & Marcotte, 2012). Therefore, future studies should include Western blotting, immunofluorescence, or targeted proteomic analyses for key proteins such as SREBP1, C/EBPβ, PPARγ, C/EBPα, and FABP4.

Second, gene disruption experiments resulted in clear phenotypic effects. Loss of *SREBF1c* and *CEBPB* expression impaired adipogenic differentiation and altered the expression of major adipogenic genes. These findings strongly support their functional role. However, the broader transcriptional consequences of these disruptions were not investigated. Future studies using transcriptome-wide approaches such as RNA-seq could help identify additional downstream targets, compensatory pathways, and regulatory networks involved in porcine adipogenesis.

Third, the epigenetic analysis focused on selected histone modifications and selected genomic regions. The analyzed marks provided useful information about chromatin activity during adipogenesis. However, histone regulation is complex, and different histone modifications may have different functional effects depending on genomic context (Bannister & Kouzarides, 2011). Future studies could include

additional marks, such as H3K27ac, H3K4me3, H3K27me3, or H3K9me3, to obtain a broader view of chromatin regulation.

Another limitation is that the ChIP-qPCR analysis was locus specific. This approach is useful for testing selected genes, but it does not capture genome-wide chromatin changes. ChIP-seq would allow broader profiling of histone marks and transcription factor occupancy across the genome (Park, 2009). This would help identify additional regulatory regions involved in porcine adipogenesis.

Finally, all experiments were performed using in vitro cell culture models. This system allows controlled analysis of adipogenic differentiation. However, it does not fully reproduce the complexity of adipose tissue in vivo. Adipose tissue contains multiple cell types, extracellular matrix components, endocrine signals, and metabolic inputs. Single-cell approaches could also help resolve cell-to-cell heterogeneity during differentiation, which cannot be fully captured by bulk analyses (Tang & Lane, 2012).

Despite these limitations, the study provides a coherent view of transcriptional and epigenetic regulation during porcine adipogenesis. Future work should combine protein-level validation, transcription factor binding assays, broader chromatin profiling, and in vivo validation to better define the molecular mechanisms controlling adipocyte differentiation.

10.6. Summary of findings

This study provides a comprehensive analysis of transcriptional and epigenetic regulation of adipogenesis in porcine cells. The results demonstrate that adipocyte differentiation is controlled by a coordinated network of transcription factors and chromatin modifications.

The functional analysis of *SREBF1* showed that the *SREBF1c* isoform is essential for adipogenic differentiation. Its disruption led to a complete inhibition of lipid accumulation and reduced expression of key adipogenic genes. These findings

confirm that *SREBF1* is not only involved in lipid metabolism but also plays a critical role in regulating adipogenic gene expression.

The results obtained for *CEBPB* indicate that it functions as an early regulator of adipogenesis. Its disruption impaired both cell proliferation and differentiation, highlighting its role in the initial stages of adipogenic commitment. The observed effects on proliferation markers suggest that *CEBPB* is required for proper progression of the early adipogenic program.

The epigenetic analysis revealed dynamic changes in histone modifications during differentiation. Increased enrichment of activating histone marks was associated with upregulation of adipogenic genes, while decreased acetylation was linked to repression of inhibitory factors. These results demonstrate that chromatin remodeling is connected to transcriptional regulation.

Importantly, the integration of transcriptional and epigenetic data shows that adipogenesis is regulated in a sequential and coordinated manner. Early transcription factors, such as *CEBPB*, are activated first, followed by downstream regulators including *PPARG* and *CEBPA*. These events are accompanied by corresponding changes in chromatin structure, indicating that epigenetic mechanisms define the timing of gene activation.

Overall, the findings of this study demonstrate that successful adipogenesis requires both proper transcription factor activity and coordinated chromatin remodeling. The results provide new insight into the molecular mechanisms controlling adipocyte differentiation and contribute to a better understanding of adipose tissue biology.

11. Conclusion

This doctoral study investigated the transcriptional and epigenetic mechanisms regulating porcine adipogenesis, with particular emphasis on the functional roles of *SREBF1* and *CEBPB* and the contribution of chromatin remodeling during adipocyte differentiation. The obtained results support the proposed hypotheses and demonstrate that adipogenesis is controlled through coordinated interactions between transcription factors and epigenetic regulatory mechanisms.

The main conclusions of this study are summarized as follows:

1. CRISPR/Cas9-mediated disruption of the *SREBF1c* regulatory region effectively impaired adipogenic differentiation, confirming the essential function of this isoform during porcine adipogenesis.
2. Disruption of the *CEBPB* promoter region affects both the proliferative capacity and adipogenic differentiation potential of porcine mesenchymal stem cells, indicating that *CEBPB* acts as an early regulator of adipogenesis and mitotic clonal expansion.
3. Histone modifications associated with adipogenic genes (*PPARG*, *GATA2*, *CEBPA*, and *CEBPB*) exhibit dynamic and stage-dependent changes during porcine adipogenesis, supporting their role in regulation during differentiation.
4. Histone modification dynamics differed between promoter and exon regions, suggesting region-specific chromatin regulation during adipocyte differentiation.
5. Sequential activation of adipogenic transcription factors is coordinated through interactions between chromatin remodeling and transcription factor activity, indicating temporal regulation of adipogenesis at both transcriptional and epigenetic levels.
6. The obtained results provide new insights into the molecular mechanisms regulating porcine adipogenesis and demonstrate the usefulness of CRISPR/Cas9 and epigenetic analyses in functional studies of adipose tissue development.

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13. List of Figures

Figure 1. Schematic representation of some obesity related diseases. Obesity contributes to a wide range of metabolic and systemic complications. *Modified* from Kyrou et al., (2015).

Figure 2. Schematic illustration of increased adiposity in pigs and its effects on metabolism, animal welfare, and production performance.

Figure 3. Adipose tissue expansion through hypertrophy and hyperplasia. *Modified from* Horwitz et al., 2023).

Figure 4. Comparison of white, brown, and beige adipose tissues. White adipose tissue (WAT) primarily stores energy as triglycerides, whereas brown adipose tissue (BAT) dissipates energy through UCP1-mediated thermogenesis. Beige adipose tissue represents an intermediate type with inducible thermogenic capacity. *Modified* from Hemadri et al., (2025).

Figure 5. Transcriptional network regulating of adipogenesis. Early regulators such as C/EBPβ initiate adipogenesis, leading to activation of PPARG, the master regulator of adipocyte differentiation. PPARG subsequently induces C/EBPα and downstream adipocyte-specific genes. SREBF1c contributes to lipid metabolism, while GATA2 acts as a negative regulator. Genes highlighted in green represent those investigated in this study. *Modified* from Zhang et al., (2020).

Figure 6. Differentiation potential of mesenchymal stem cells (MSCs) into multiple cell lineages, including adipocytes. *Modified* from James, (2013).

Figure 7. Overview of epigenetic mechanisms regulating gene expression. DNA methylation, histone modifications, and non-coding RNAs control chromatin structure and gene activity, leading to gene activation or repression.

Figure 8. Distribution of histone modifications associated with active and silent gene states. Activating histone marks are enriched in transcriptionally active regions,

whereas repressive marks are associated with gene silencing. *Modified* from Lim et al., (2010).

Figure 9. Schematic representation of the CRISPR-Cas immune system. The figure illustrates the stages of foreign DNA acquisition, crRNA biogenesis, and RNA-guided interference. Previously published by the author Rozynek et al., (2023).

Figure 10. CRISPR/Cas9 gene editing mechanism. Cas9 guided by RNA induces a double-strand break, repaired by NHEJ or HDR pathways. Previously published by the author Rozynek et al., (2023).

Figure 11. Schematic representation of adipogenic differentiation in mesenchymal stem cells (MSCs) over time. Cells show gradual lipid droplet accumulation from day 0 to day 10, indicating the transition from undifferentiated MSCs to mature adipocytes.

Figure 12. Workflow of lipid accumulation analysis using BODIPY staining. Cells were fixed with paraformaldehyde, washed, and stained with BODIPY 493/503 to visualize intracellular lipid droplets, followed by DAPI counterstaining for nuclei. Fluorescence microscopy was used to assess lipid accumulation during adipogenic differentiation.

Figure 13. Workflow of CRISPR/Cas9 gene editing experiments in mesenchymal stem cells (MSCs). The process includes gRNA design, plasmid construction and its isolation, transfection of MSC via nucleofection, followed by selection of single-cell MSC colonies and downstream validation by qPCR and genotyping.

Figure 14. Workflow of CRISPR/Cas9 genotyping and validation. Genomic DNA isolated from single-cell-derived MSC colonies was amplified by PCR and analyzed by agarose gel electrophoresis to detect deletion events. PCR products were purified and subjected to Sanger sequencing, followed by alignment with the reference genome to confirm targeted modifications.

Figure 15. Workflow of gene expression analysis during adipogenesis. Cells undergoing adipogenic differentiation were subjected to RNA isolation, followed by

cDNA synthesis using reverse transcription, and analyzed by quantitative PCR (qPCR) to assess gene expression levels.

Figure 16. Workflow of chromatin immunoprecipitation (ChIP) followed by qPCR analysis during adipogenesis. Cells were crosslinked to preserve protein–DNA interactions, chromatin was sheared, and target complexes were immunoprecipitated using specific antibodies. After reverse crosslinking and DNA purification, enrichment of target regions was analyzed by qPCR.

Figure 17. Schematic summary of the major findings on *SREBF1* function in pigs.

Figure 18. Schematic summary of the major findings on *CEBPB* function in pigs.

Figure 19. Schematic summary of histone modifications.

Scientific Achievements

List of Publications (Original papers):

1. **Aksoy, M. O.,** Wozniak, J., Stachowiak, M., & Szczerbal, I. (2026). Dynamic Histone Modification Patterns in Key Transcription Factor Genes During Porcine Adipogenesis. *Genes*, *17*(5), 521. MNiSW points=100, 5-year Impact Factor= 3.2

2. **Aksoy, M. O.,** Rozynek, J., Stachowiak, M., & Szczerbal, I. (2026). The role of the CEBPB gene in porcine adipogenesis: a study using CRISPR/Cas9-edited mesenchymal stem cells. *Journal of Applied Genetics*, 67, 447-486. MNiSW points=140, 5-year Impact Factor=2.0

3. Loba-Pasternak, W., **Aksoy, M. O**., Stuper-Szablewska, K., Szwajkowska-Michalek, L., Kolodziejski, P., Szczerbal, I., & Nowacka-Woszuk, J. (2025). The Effects of Peruvian maca (Lepidium meyenii) Root Extract on In Vitro Cultured Porcine Fibroblasts and Adipocytes. *Molecules*, *30*(4), 847. MNiSW points=140, 5-year Impact Factor=5.0

4. **Aksoy, M. O**., Bilinska, A., Stachowiak, M., Flisikowska, T., & Szczerbal, I. (2024). Deciphering the Role of the SREBF1 Gene in the Transcriptional Regulation of Porcine Adipogenesis Using CRISPR/Cas9 Editing. *International Journal of Molecular Sciences*, *25*(23), 12677. MNiSW points=140, 5-year Impact Factor=5.7

5. Yurttaş, L., Evren, A. E., Kubilay, A., **Aksoy, M. O**., Temel, H. E., & Akalın Çiftçi, G. (2023). Synthesis of some new 1, 3, 4-oxadiazole derivatives and evaluation of their anticancer activity. *ACS omega*, *8*(51), 49311-49326. MNiSW points=70, 5-year Impact Factor=4.4

6. Kaya, B., Yurttaş, L., Akalın-Çiftçi, G., & **Aksoy, M. O.** (2023). Design, synthesis and apoptotic effects of novel benzoxazole compounds. *Zeitschrift für*

Naturforschung C, *78*(11-12), 433-440. MNiSW points=40, 5-year Impact Factor=2.1

7. Bilinska, A., Pszczola, M., Stachowiak, M., Stachecka, J., Garbacz, F., **Aksoy, M. O**., & Szczerbal, I. (2023). Droplet digital PCR quantification of selected intracellular and extracellular microRNAs reveals changes in their expression pattern during porcine in vitro adipogenesis. *Genes*, *14*(3), 683. MNiSW points=100, 5-year Impact Factor=3.2

8. Szczerbal, I., Malek, E., Rigillo, A., Lukomska, A., Kacprzak, K., Gasparini, S., Nowacka-Woszuk, J., Stachowiak, M., **Aksoy, M. O.,** Switonski, M. (2023). Non-mosaic X monosomy (77, X) in a female dog with signs of virilization. *Journal of Applied Genetics*, *64*(1), 169-172. MNiSW points=140, 5-year Impact Factor=2.0

9. Dincel, E. D., Akdağ, Ç., Kayra, T., Coşar, E. D., **Aksoy, M. O**., Akalın-Çiftçi, G., & Ulusoy-Güzeldemirci, N. (2022). Design, synthesis, characterization, molecular docking studies and anticancer activity evaluation of novel hydrazinecarbothioamide, 1, 2, 4-triazole-3-thione, 4-thiazolidinone and 1, 3, 4-oxadiazole derivatives. *Journal of Molecular Structure*, *1268*, 133710. MNiSW points=70, 5-year Impact Factor=5.0

10. Yurttaş, L., Temel, H. E., **Aksoy, M. O**., Bülbül, E. F., & Çiftçi, G. A. (2022). New chromanone derivatives containing thiazoles: Synthesis and antitumor activity evaluation on A549 lung cancer cell line. *Drug Development Research*, *83*(2), 470-484. MNiSW points=70, 5-year Impact Factor=3.9

11. Evren, A. E., Yurttaş, L., Ekselli, B., **Aksoy, M. O**., & Akalin-Çiftçi, G. (2021). Design and efficient synthesis of novel 4, 5-dimethylthiazole-hydrazone derivatives and their anticancer activity. *Letters in Drug Design & Discovery*, *18*(4), 372-386. MNiSW points=20, 5-year Impact Factor=1.2

12. Yurttaş, L., Çiftçi, G. A., **Aksoy, M. O**., & Demirayak, Ş. (2020). Novel benzimidazole derivatives: Cytotoxic and apoptotic properties on lung cancer cell

line. *Letters in Drug Design & Discovery*, *17*(10), 1227-1236. MNiSW points=20, 5-year Impact Factor=1.2

Popular Science Article:

1. Jędrzej Rozynek, **Mehmet O. Aksoy**, Izabela Szczerbal (2023). Możliwości wykorzystania techniki CRISPR/Cas9 w hodowli świń. *Przegląd hodowlany,* 3/2023. (Popular Science)

Conferences:

1. 30th Genetic Days, 2024. Książ Castle/Poland

   Aksoy M.O., Stachowiak M., Szczerbal I. (2024), The role of histone modifications during adipocyte differentiation in the pig., XXX Genetic Days, 10-13 September 2024, Książ, Poland. (Poster presentation)

   Rozynek J., **Aksoy M.0**., Stachowiak M., Szczebal I. (2024), Application of CRISPR/Cas9 method for editing the CEBPB locus in porcine mesenchymal stem cells (pMSC)., XXX Genetic Days, 10-13 September 2024, Książ, Poland. (Poster presentation)

2. 25th International Colloquium on Animal Cytogenetics and Genomics, 2024. Naples/Italy

   Aksoy M.O., Stachowiak M., Izabela Szczerbal I. (2024), Alterations in Histone Modifications in the Genomic Region Harboring Genes Encoding Transcription Factors Crucial for Adipocyte Differentiation in the Pig., 25th International Colloquium on Animal Cytogenetics and Genomics, 26–29 June 2024, Naples, Italy. Biol. Life Sci. Forum 2024, 33, 1. (Poster presentation)

Rozynek R., **Aksoy M.O**., Stachowiak M., Szczerbal I. (2024), Use of Crispr/Cas9 Genome Editing to Investigate the Role of the CEBPB Gene during In Vitro Adipogenesis in the Pig., 25th International Colloquium on Animal Cytogenetics and Genomics, 26–29 June 2024, Naples, Italy. Biol. Life Sci. Forum 2024, 33, 1. (Poster presentation)

3. 6th Polish Genetics Congress, 2022. Krakow/Poland

   Aksoy M.O,. Stachowiak M., Szczerbal I. (2022), Analysis of histone modifications in porcine genomic regions harboring two genes encode transcription factors for adipogenesis – *PPARG* and *GATA2* by chromatin immunoprecipitation (ChIP) method., 6th Polish Congress of Genetics 27-30.06.2022 Kraków, Poland (Poster presentation)

   Bilinska A., Stachowiak M., **Aksoy M.O.,** Szczerbal I. (2022), Generation of porcine mesenchymal stem cells (pMSC) with a knockout of the *SREBF1* gene using the CRISPR-Cas9 system., 6th Polish Congress of Genetics 27-30.06.2022 Kraków, Poland (Poster presentation)

   Szczerbal, I.,Lukomska, A., Kacprzak, K., Nowacka-Woszuk, J., Stachowiak, M., **Aksoy, M. O**., Malek, E., Switonski, M. Non-mosaic X monosomy in a female dog with abnormal sexual development., 6th Polish Congress of Genetics 27-30.06.2022 Kraków, Poland (Poster presentation)

4. 31st National Biochemistry Congress, 2020. TBS Online/ Turkey

   Çiftçi, G. A., Temel, H. E., Evren, A. E., **Aksoy, M. O**., Kubilay A., Yurttas L., (2020) Design, Synthesis, and Investigation of Anticancer Activities of New Compounds Bearing Oxadiazole-Acetamide Structure., 31st National Biochemistry Congress 18-20.12.2020, TBS Online/ Turkey (Oral presentation)

5. 12th International Symposium on Pharmaceutical Sciences, 2018. Ankara/Turkey

   Aksoy, M. O., Çiftçi, G. A. (2018) Synthesis and Evaluation of New 2-Substitued Benzoxazole Derivatives as Anticancer Agents., 12th International Symposium

on Pharmaceutical Sciences, June 26-29, 2018., Ankara/Turkey (Poster presentation)

6. 5th International Conference on Computation for Science and Technology, 2018. Antalya/Turkey

   Yurttaş L., **Aksoy M. O.,** Akalin Çiftçi G., Demirayak S., (2018) New Thiocarbamoyl-Benzimidazole Derivatives as Potentical Antitumor Agents., 5th International Conference on Computation for Science and Technology , 23-26 September 2018 (Poster Presentation)

Projects:

1. The application of CRISPR-Cas9 technology to elucidate whether changes in nuclear architecture during adipogenesis are a cause or a consequence? Scholarship holder, OPUS project, 2021-2025. Funded by National Science Center, Poland
2. Investigation of Biochemical Action Mechanisms of Some Chalcone Derivatives. Researcher, MSc thesis project, 2018-2020. Funded by Anadolu University Scientific Project Center, Turkey
3. Synthesis of Some New Chromium-Thiazole Derivatives and Investigation of Anticancer Activities. Researcher, Research Publication Incentive Project, 2019-2020. Funded by Anadolu University Scientific Project Center, Turkey
4. Synthesis of New 1,3,4-Oxadiazole Derivatives Carrying Acetamide Residue and Investigation of Anticancer Activities. Researcher, Research Publication Incentive Project, 2018-2019. Funded by Anadolu University Scientific Project Center, Turkey

5. Pharmacognostic Investigation of Some Citrus Species. Scholarship holder, Research Publication Incentive Project, 2018-2019. Funded by Anadolu University Scientific Project Center, Turkey

Internships:

1. Erasmus+ Internship Program: 2020 (5 months)

   Polish Academy of Sciences, Institute of Human Genetics, Department of Nucleic Acid Funtion. Poznan/Poland

   Supervisor: Prof . Dr . Hab . Jadwiga Jaruzelska

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(According to Google Scholar)

Number of original publications: 12

Number of popular science articles: 1

Citations: 139

Hirsch index: 6

Total Impact Factor: 38.9

Total MNiSW: 1050

Appendix A – Published Article I

Aksoy, M. O., Bilinska, A., Stachowiak, M., Flisikowska, T., & Szczerbal, I. (2024). Deciphering the role of the *SREBF1* gene in the transcriptional regulation of Porcine adipogenesis using CRISPR/Cas9 editing. *International Journal of Molecular Sciences*, 25(23), 12677. https://doi.org/10.3390/ijms252312677

MNiSW points= 140, 5-year Impact Factor= 5.7

Appendix B – Published Article II

Aksoy, M. O., Rozynek, J., Stachowiak, M., & Szczerbal, I. (2026). The role of the *CEBPB* gene in porcine adipogenesis: a study using CRISPR/Cas9-edited mesenchymal stem cells. *Journal of Applied Genetics*, 67,447-486**.**
https://doi.org/10.1007/s13353-025-01030-x

MNiSW points=140, 5-year Impact Factor= 2.0

Appendix C – Published Article III

Aksoy, M. O., Wozniak, J., Stachowiak, M., & Szczerbal, I. (2026). Dynamic Histone Modification Patterns in Key Transcription Factor Genes During Porcine Adipogenesis. *Genes*, *17*(5), 521. https://doi.org/10.3390/genes17050521

MNiSW points= 100, 5-year Impact Factor= 3.2

International Journal of
Molecular Sciences

MDPI

Article

Deciphering the Role of the *SREBF1* Gene in the Transcriptional Regulation of Porcine Adipogenesis Using CRISPR/Cas9 Editing

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Abstract: Sterol regulatory element-binding protein 1 (SREBP1) is an important transcription factor that controls lipid metabolism and adipogenesis. Two isoforms, SREBP1a and SREBP1c, are generated by alternative splicing of the first exon of the *SREBF1* gene. The porcine *SREBF1* gene has mainly been studied for its role in lipid metabolism in adipose tissues, but little is known about its involvement, and the role of its two isoforms, in adipogenesis. The aim of the present study was to introduce a deletion in the 5′-regulatory region of the *SREBF1c* gene, considered crucial for adipogenesis, using the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9) method. This approach allows for the evaluation of how inhibiting *SREBF1c* transcription affects the expression of other genes essential for adipocyte differentiation, particularly *PPARG*, *CEBPA*, *CEBPB*, *CEBPD*, *GATA2*, and *FABP4*. It was observed that disrupting the *SREBF1c* isoform had no effect on the *GATA2* gene but did result in a decrease in the expression of the *CEBPA* and *CEBPD* genes, an increase in the expression of *CEBPB*, and an inhibition in the expression of the *PPARG* and *FABP4* genes. These changes in gene expression blocked adipogenesis, as could be seen by the failure of lipid droplets to accumulate. Our results provide evidence highlighting the pivotal role of the *SREBP1c* isoform in the regulation of porcine adipogenesis.

Keywords: adipogenesis; CRISPR/Cas9; isoforms; MSC; pig; *SREBF1*; SREBP1

Citation: Aksoy, M.O.; Bilinska, A.; Stachowiak, M.; Flisikowska, T.; Szczerbal, I. Deciphering the Role of the *SREBF1* Gene in the Transcriptional Regulation of Porcine Adipogenesis Using CRISPR/Cas9 Editing. *Int. J. Mol. Sci.* **2024**, *25*, 12677. https://doi.org/10.3390/ijms252312677

Academic Editor: Melvin K. S. Leow

Received: 2 November 2024
Revised: 23 November 2024
Accepted: 24 November 2024
Published: 26 November 2024

1. Introduction

Sterol regulatory element binding proteins (SREBPs) are a family of basic helix–loop–helix leucine zipper transcription factors. They regulate lipid homeostasis by binding to sterol regulatory elements (SREs) in target genes. Two proteins, SREBP1 and SREBP2, are encoded by the two genes *SREBF1* and *SREBF2*, respectively. The SREBP1 protein is involved in the synthesis of fatty acid, phospholipid, and triacylglycerol, while SREBP2 is responsible for the regulation of cholesterol metabolism [1,2]. Two isoforms of SREBP1, called SREBP1a and SREBP1c, have been recognized. They arise from alternative transcription start sites and differ in their first exon [3]. The SREBP1c isoform is the predominant form expressed in in liver, adipose tissue, and skeletal muscle, whereas expression of SREBP1a has been found in the spleen, small intestine, heart, thymus, and proliferating cell lines [4,5]. SREBP2 is ubiquitously expressed in different tissues, especially in embryonic tissues, liver, and adipose tissue [6,7].

The SREBPs have been extensively studied in rodents, in which lipogenesis is regulated in both the liver and adipose tissue, while, in nonrodent mammals such as the domestic pig, only one lipogenic organ is active—the adipose tissue [8]. SREBP1 has been recognized as a key regulator of lipogenesis in pigs [9]. The *SREBF1* gene plays a role in fat metabolism and intramuscular fat content in pigs. It has been shown that the expression of the *SREBF1*

gene correlates with the fat deposition rate in growing pigs [10]. It is also important for the regulation of fat deposition in muscle during the postnatal growth of pigs [11]. Higher expression levels of the *SREBF1* gene have been observed in the muscle tissues of fatty pig breeds compared to those in the leaner pig [12]. Polymorphisms in the *SREBF1* gene have been associated with fatness traits in pigs [13–15].

SREBP1 is also involved in the regulation of adipogenesis. Initially, it was named "adipocyte determination and differentiation-dependent factor 1" (ADD1), which was later identified as the SREBP1c isoform [16,17]. The process of fat cells formation is controlled by a complex transcriptional network of many factors, of which proliferator-activated receptor γ (PPARγ) and members of the CCAAT-enhancer-binding protein (CEBP), such as C/EBPα and C/EBPβ, play a central role [18] (Figure 1). SREBP1 was identified as a proadipogenic transcription factor that induces PPARγ expression through the production of endogenous ligands [16]. The role of SREBP1 in adipogenesis has mainly been studied in mouse and human cells. There have been a number of studies suggesting that the *Srebf1c* isoform is the main transcription factor of adipogenesis [18–20]. The expression of this isoform increases during differentiation of the cultured mouse 3T3-L1 preadipocytes. On the other hand, studies in transgenic mice overexpressing SREBP1c have shown that adipocyte differentiation is inhibited, leading to lipodystrophy [21]. It has also been shown that the amount of white adipose tissue was not significantly decreased in mice with the disrupted *Srebp1c* gene [22]. Subsequent experiments on 3T3-F442A preadipocytes have revealed that the *Srebf1a* isoform is also a key regulator of transcriptional cascade during adipogenesis [23]. The loss of function of *Srebf1a* inhibits adipocyte differentiation. Research on *SREBF1* isoforms has also been extended to human cells: using the Simpson–Golabi–Behmel syndrome (SGBS) preadipocyte cell line as a valuable model for studying human adipocyte function [24]. RNA interference experiments in SGBS cells showed that while both SREBP1 variants were targeted, only SREBP1a expression significantly decreased [25]. The SREBP1a isoform was predominant in these cells, and SREBP1c was expressed at a low level. These studies show differences in the functioning of the SREBP1 transcription factor depending on whether cells or model organisms are used.

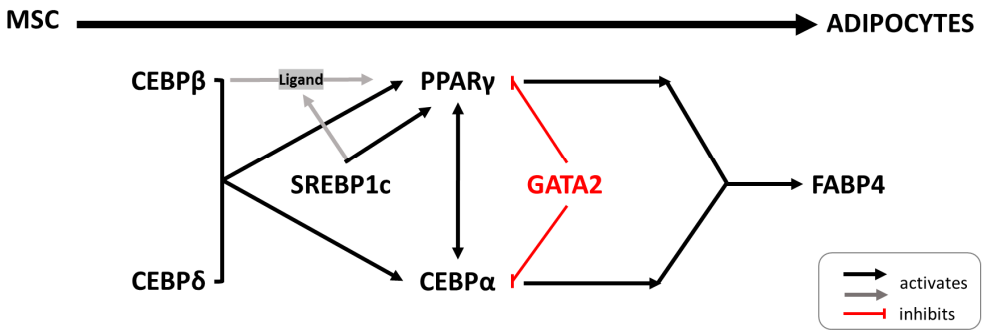

Figure 1. Simplified scheme of transcriptional regulation in adipogenesis, highlighting the transcription factors analyzed in this study. Adapted from [18].

Data on the role of the *SREBF1* gene during adipogenesis in the pig are scarce, despite the importance of understanding the mechanisms controlling fat tissue formation in this key livestock species [26]. Adipose tissue grows by two processes: the generation of new adipocyte cells and the increase in the size of adipocytes [27]. Adipose tissue impacts neonatal survival, reproductive ability, postnatal growth, and meat production efficiency [28]. The pig has also been recognized as a better animal model for human obesity than rodents due to anatomical, physiological, and metabolic similarities with humans [29,30]. Regarding the role of the *SREBF1* gene during porcine adipogenesis, studies have only demonstrated that its expression levels increase progressively with the duration of differentiation [31,32]. Information on the role of the two *SREBF1* isoforms is also lacking.

Given the limited data on the role of the *SREBF1* gene and the significance of its two transcriptional forms in porcine adipogenesis, our study aimed to explore the pivotal

role of this transcription factor in pigs. The revolutionary gene-editing tool CRIPSR/Cas9 has enabled the generation of gene-specific knockouts which can be used to study gene function [33]. To clarify the role of the *SREBF1* gene during adipogenesis in the pig, we generated a mesenchymal stem cells derived from adipose tissue (AD-MSC) with a targeted deletion in the *SREBF1* gene using the CRIPSR/Cas9 method. This deletion disrupts the 5′-regulatory region specific to the *SREBF1c* isoform while preserving the *SREBF1a* isoform, as the deletion was localized in an intron. Analysis of the transcriptional activity of the *SREBF1* gene in relation to other key genes that are important in adipocyte differentiation (*PPARG*, *CEBPA*, *CEBPB*, *CEBPD*, *GATA2*, and *FABP4*) allowed us to describe their expression patterns during differentiation. Understanding *SREBF1* activity provides valuable insights into the timing and sequential activation of key genes involved in adipocyte differentiation.

2. Results

2.1. Characterization of the 5′-Regulatory Region in Porcine SREBF1 and the Introduction of a CRISPR/Cas9-Mediated Deletion in This Region

Since the promoter region of the porcine *SREBF1* gene has not been well characterized, we performed, as the first step, an in silico analysis of the 5′-regulatory sequence by identifying binding sites for known transcription factors for adipogenesis using DNA Star Lasergene (https://www.dnastar.com/software/ accessed on 10 January 2022). Several regulatory elements—such as TATA-box, SP1 elements, SRE/SRE-2, NF-Y, and LXRE1 motifs—were identified in the proximal 5′-regulatory region of the gene that encodes the *SREBF1c* isoform. To delete the proximal regulatory region harboring the majority of regulatory elements, four single guide RNAs (sgRNAs) were tested in different combinations, but, ultimately, two were selected for introducing cleavage sites in porcine mesenchymal stem cells derived from adipose tissue (AD-MSC) (Figure 2, Table S1).

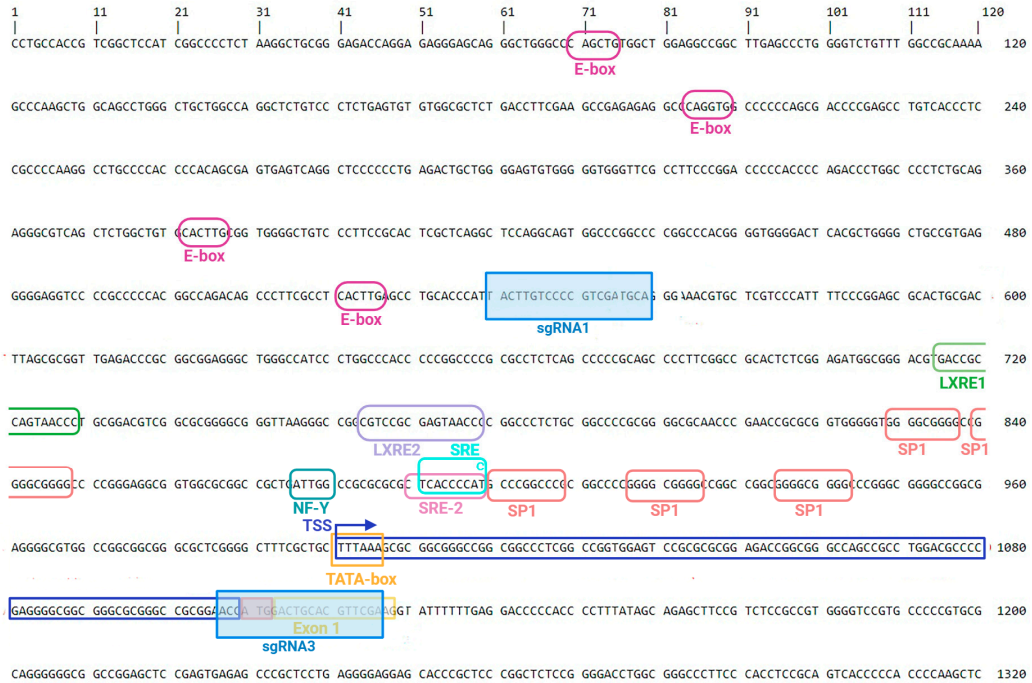

Figure 2. The 5′-regulatory sequence of *SREBF1c* with marked transcription factor binding sites and positions of two sgRNAs (sgRNA1 and sgRNA3 in blue frames) used to excise the proximal promoter region.

sgRNA1 and sgRNA3 were cloned into the pX330 plasmid, and AD-MSCs were transfected via nucleofection. The transfection efficiency of AD-MSCs, measured with a control plasmid (pmaxGFP Vector, Lonza, Basel, Switzerland), was approximately 40% (Figure S1). Single AD-MSC colonies post-transfection were genotyped by PCR, targeting

regions overlapping the sgRNA binding sites, resulting in the identification of two colonies with deletions in the target gene (Figure S2). Subsequent Sanger sequencing of PCR products from these cells revealed a 567 bp deletion in the 5′-regulatory region of *SREBF1c* (Figure 3). In silico analysis of the porcine *SREBF1* gene sequence confirmed that this deleted fragment was located within intron 1 of the *SREBF1a* isoform (Figure 4). This approach enabled us to obtain modified AD-MSCs with a non-functional *SREBF1c* isoform while preserving an intact transcript of the *SREBF1a* isoform.

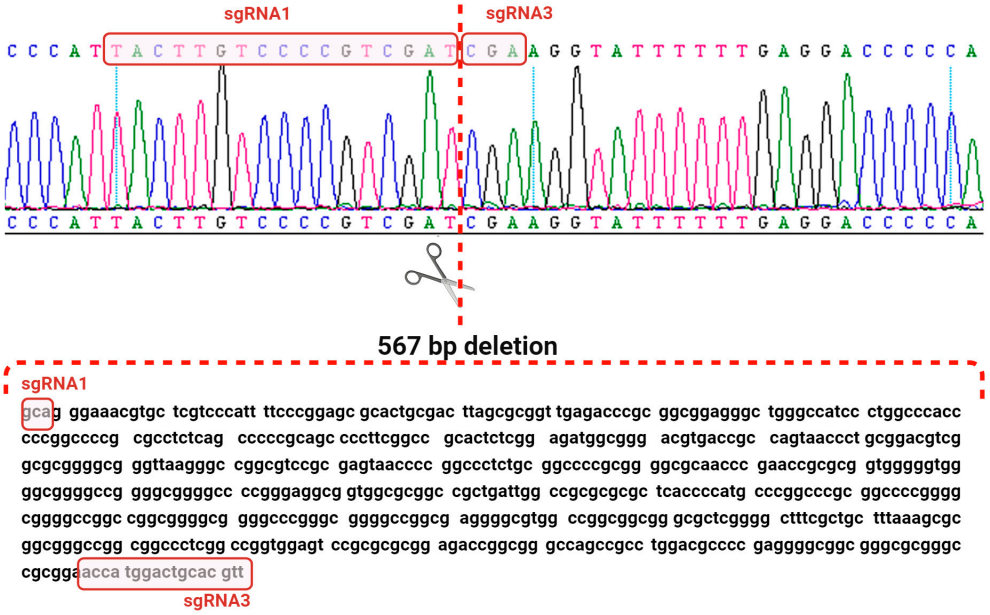

Figure 3. Sanger sequencing of the PCR product confirmed the deletion of 567 bp in the *SREBF1c* 5′-regulatory region. The position of the joined DNA fragments in the AD-MSC cells and the partial sequences recognized by sgRNA1 and sgRNA3 are shown.

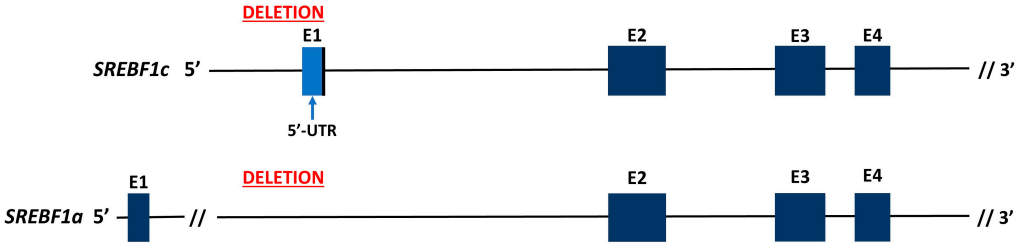

Figure 4. Location of the 567 bp deletion site in the genomic region covering *SREBF1a* and *SREBF1c* isoforms according to Sscrofa 11.1 assembly (NC_010454.4) chromosome 12 reference sequence. E1–E4: exons 1–4. Genomic position of the 567 bp deletion: 12: 60740868-60741434.

2.2. Characteristics of the Expression Profile of the SREBF1a and of SREBF1c Isoforms in Wide-Type Cells (AD-MSC$_{WT}$) and in AD-MSC with Disrupted SREBF1c Gene (AD-MSC$_{DEL}$) and Adipose Tissues

The relative transcript level of the *SREBF1a* isoform was similar in undifferentiated cells (day 0) for both AD-MSC$_{WT}$ or AD-MSC$_{DEL}$ (Figure 5a). After induction of adipogenesis, increased expression of this isoform was detected on day 2 in AD-MSC$_{WT}$, with its level being significantly higher than in AD-MSC$_{DEL}$ ($p < 0.001$). In days 6 and 8 of differentiation, a higher level of *SREBF1a* was observed in AD-MSC$_{DEL}$ ($p < 0.05$ for day 6 and $p < 0.001$ for day 8). The analysis of the transcript level of *SREBF1c* in AD-MSC$_{WT}$ indicated that there was an increase in expression from day 8 of differentiation (Figure 5b). As expected, no expression of this isoform was detected in AD-MSC$_{DEL}$.

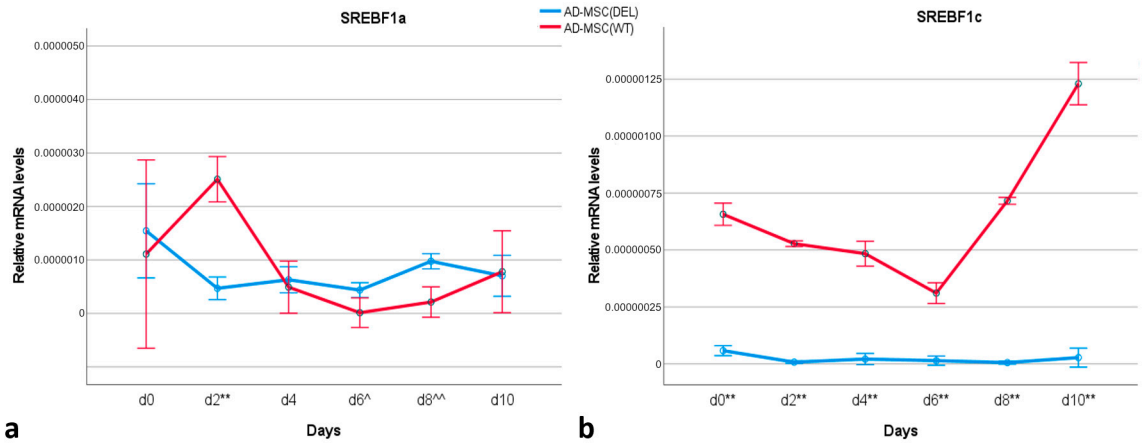

Figure 5. Relative transcript levels of *SREBF1a* (**a**) and *SREBF1c* (**b**) during subsequent days of adipogenesis. The error bars represent 95% confidence intervals. **: significantly higher in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$, $p < 0.001$; ^^: significantly lower in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$, $p < 0.001$; ^: significantly lower in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$, $p < 0.05$.

Additionally, the expression of both isoforms of the *SREBF1* gene was analyzed in porcine adipose tissue to determine which isoform plays an essential role in the function of this tissue in pigs. Two adipose tissue were analyzed—subcutaneous and visceral. In both tissues, the expression of *SREBF1c* was significantly higher than in *SREBF1a* (Figure S3). Both transcriptional forms showed higher expression in visceral fat tissues ($p < 0.001$) (Figure 6).

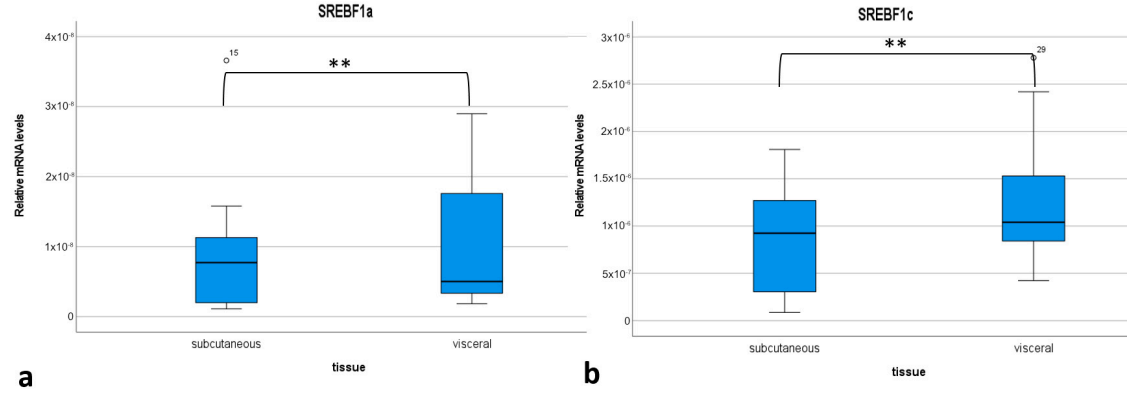

Figure 6. Relative mRNA levels of *SREBF1a* (**a**) and *SREBF1c* (**b**) isoforms in the subcutaneous and visceral adipose tissue of wild-type pigs. The boxplots show the lower quartile (lower edge of the box), median (black line dividing the box into two parts), upper quartile (upper edge of the box), outliers (dots), and whiskers (values inside the lower and upper quartiles). **: significant differences between groups, $p < 0.001$, Wilcoxon's signed rank test.

2.3. Monitoring of Lipid Droplet Accumulation in AD-MSC$_{WT}$ and in AD-MSC$_{DEL}$

The accumulation of lipids was monitored using BODIPY staining for ten days of adipogenesis (Figure 7). On day 0 of differentiation (MSC), no lipid droplets were observed. On day 2 (d2), differentiation of single cells was visible in both AD-MSC$_{WT}$ and AD-MSC$_{DEL}$, but the amount of lipid droplets was higher in AD-MSC$_{WT}$ ($p < 0.05$). With day 4 of adipogenesis, a significant increase ($p < 0.001$) in lipid droplet accumulation was observed in AD-MSC$_{WT}$, while cells with a disrupted *SREBF1c* isoform (AD-MSC$_{DEL}$) did not accumulate lipid droplets and did not differentiate into adipocytes.

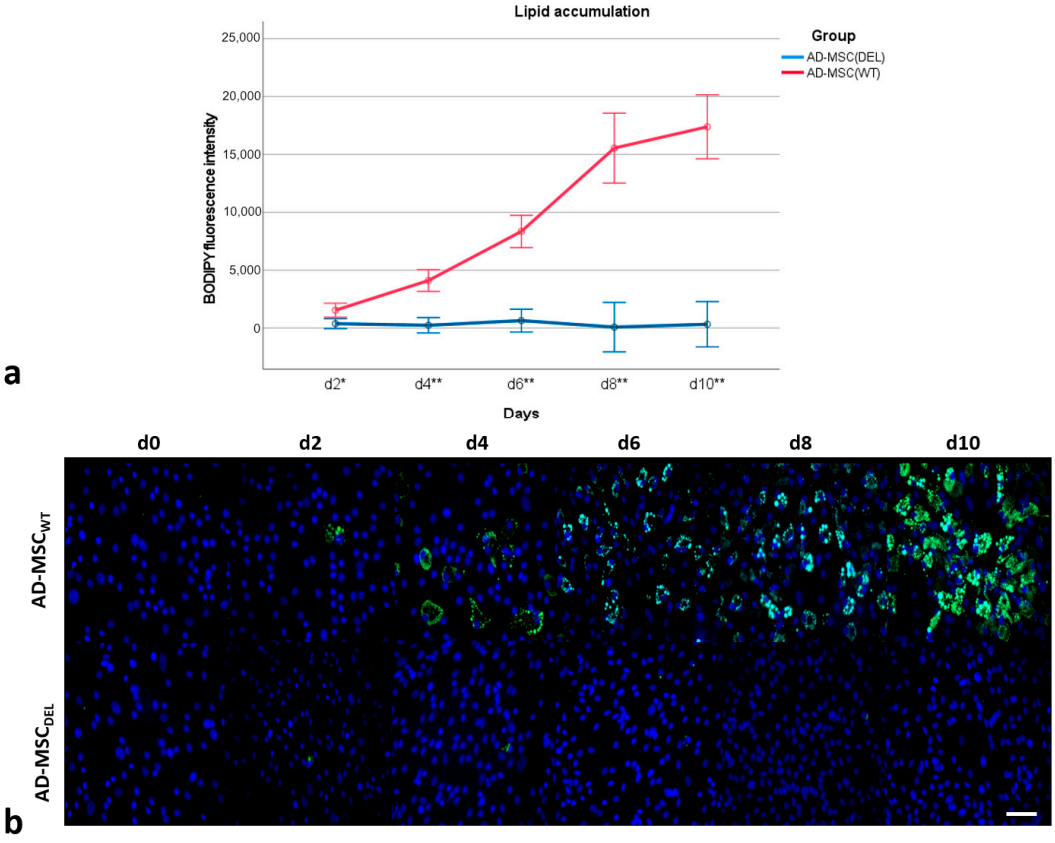

Figure 7. Measuring of lipid accumulation based on BODIPY 493/503 fluorescent intensity during successive days of adipocyte differentiation (**a**). Error bars represent 95% confidence intervals. D: day; **: significantly higher in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$, $p < 0.001$; *: significantly higher in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$, $p < 0.05$. Representative images showing adipocyte differentiation (**b**). Lipid droplets were stained with BODIPY 493/503 (green); the nuclei were counterstained with DAPI (blue). Scale bar: 50 µm.

2.4. Expression of Key Adipogenic Genes in AD-MSC$_{WT}$ and AD-MSC$_{DEL}$

The expression patterns of six genes important for adipogenesis were evaluated in AD-MSC$_{WT}$ and AD-MSC$_{DEL}$. These genes were selected as key transcription factors involved in adipogenesis (*PPARG*, *CEBPA*, *CEBPB*, *CEBPD*, and *GATA2*) and as a marker gene for adipocytes (*FABP4*).The *GATA2* gene was selected as an anti-adipogenic transcription factor, *CEBPD* and *CEBPB* as early genes induced during adipogenesis, *CEBPA* and *PPARG* as critical transcription factors in adipogenesis, and *FABP4* as a gene important for terminal adipocyte differentiation. *GATA2* was downregulated during adipocyte differentiation in both AD-MSC$_{WT}$ and AD-MSC$_{DEL}$, and its transcript level was not significantly different between these cell types apart from on day 6 ($p < 0.05$) (Figure 8a). The expression profile of *CEBPD* was very similar in AD-MSC$_{WT}$ and AD-MSC$_{DEL}$, with visibly higher expression in AD-MSC$_{WT}$ on day 0 ($p < 0.001$), day 4 ($p < 0.01$), and day 8 ($p < 0.01$) (Figure 8b). *CEBPB* showed a characteristic peak of transcriptional activity on day 4 ($p < 0.001$) of adipogenesis in AD-MSC$_{WT}$. *CEBPB* was the only gene to show a higher transcript level in AD-MSC$_{DEL}$ ($p < 0.05$) than in AD-MSC$_{WT}$ (Figure 8c). *CEBPA* was upregulated during differentiation in AD-MSC$_{WT}$, and its transcript level was significantly higher in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$ on day 2 ($p < 0.01$), day 6, day 8, and day 10 ($p < 0.001$) (Figure 8d). Increasing transcript levels of *PPARG* and *FABP4* genes were observed during differentiation in AD-MSC$_{WT}$ (Figure 8e,f). An increase in *PPARG* gene expression was observed over the subsequent days of examination, whereas *FABP4* gene expression was detected starting from day 6 of differentiation. No expression of either gene was found in AD-MSC$_{DEL}$ ($p < 0.001$).

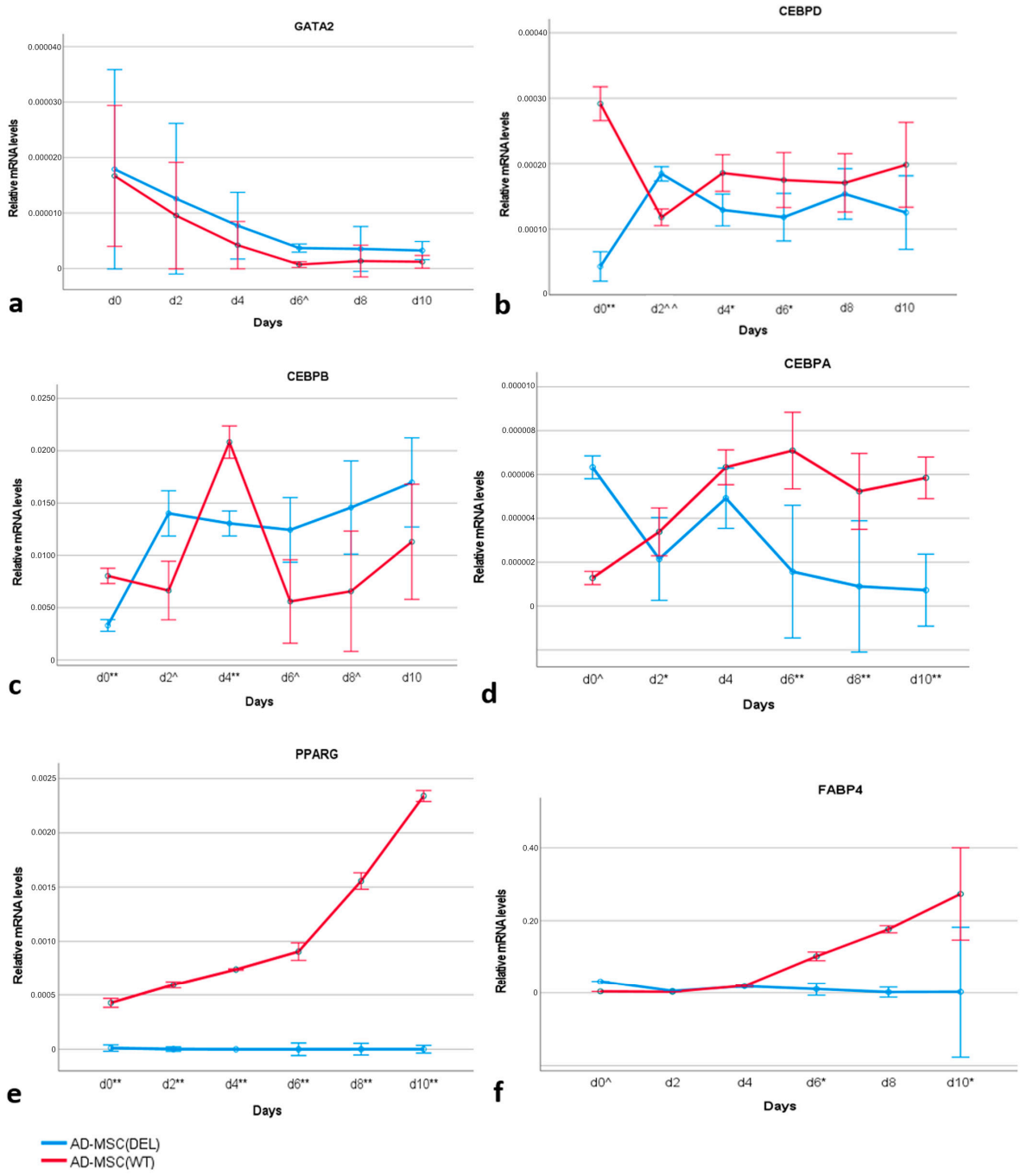

Figure 8. Relative transcript levels of *GATA2* (**a**), *CEBPD* (**b**), *CEBPB* (**c**), *CEBPA* (**d**), *PPARG* (**e**), and *FABP4* (**f**) during subsequent days of adipogenesis in AD-MSC$_{WT}$ and AD-MSC$_{DEL}$. Error bars represent 95% confidence intervals. **: significantly higher in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$, $p < 0.001$; *: significantly higher in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$, $p < 0.01$; ^^: significantly lower in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$, $p < 0.001$; ^: significantly lower in AD-MSC$_{WT}$ than in AD-MSC$_{DEL}$, $p < 0.05$.

Summarizing this experiment (Table 1), we showed that the lack of activity of *SREBF1c* resulted in adipogenesis being blocked and in the *PPARG* and *FABP4* genes not being expressed. The expression levels of *CEBPA* and *CEBPD* genes were reduced. The modification did not cause major changes in *GATA2* gene expression—instead, an increase in the expression of the *CEBPB* gene was observed in the modified AD-MSC. Since PPARγ activation relies on lipid-derived ligands produced through SREBF1c activity, the absence of the *SREBF1c* transcript led to disrupted expression of *PPARG* and, consequently, its

downstream target, *FABP4*. The disruption of the *SREBF1c* isoform affected the expression of genes acting upstream in the adipogenesis cascade, resulting in an altered expression pattern in AD-MSC$_{DEL}$.

Table 1. Summary of the results of lipid accumulation (BODIPY) and gene expression analysis in AD-MSC$_{DEL}$ when compare to AD-MSC$_{WT}$. An arrow pointing down (↓) indicates lower expression, an arrow pointing up (↑) indicates higher expression, and an equal sign (=) indicates no difference between the two cell types; - means there was no detection on that day.

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
BODIPY	-	↓	↓	↓	↓	↓
SREBF1a	=	↓	=	↑	↑	=
SREBF1c	↓	↓	↓	↓	↓	↓
GATA2	=	=	=	↑	=	=
CEBPD	↓	↑	↓	↓	=	=
CEBPB	↓	↑	↓	↑	↑	=
CEBPA	↑	↓	=	↓	↓	↓
PPRAG	↓	↓	↓	↓	↓	↓
FABP4	↑	=	=	↓	=	↓

3. Discussion

Adipocyte differentiation is governed by the complex action of many transcription factors. It has been well established that PPARγ is the master regulator of adipogenesis and that the members of the C/EBP family play critical roles in the normal course of this process. Studies of the importance of SREBP and their isoforms during adipogenesis have provided different results, and there are no data about the role of these isoforms in the formation of fat cells in the pig. In the present study, we used the CRIPSR/Cas9 genome editing technique to reveal the crucial role of the *SREBF1c* gene in adipogenesis in the pig. The study was performed on a well-established system of in vitro differentiation of mesenchymal stem cells into adipocytes [34,35]. Temporal changes in the transcript level of seven genes (*SREBF1*, *PPARG*, *CEBPA*, *CEBPB*, *CEBPD*, *GATA2*, and *FABP4*) were detected in both wild-type and modified AD-MSCs. The transcriptional profiles of the genes in AD-MSC$_{WT}$ were similar to those observed during adipocyte differentiation of 3T3-L1 cells and to experiments performed previously on the porcine in vitro adipogenesis system [31]. As a negative regulator of adipogenesis, *GATA2* was downregulated during porcine adipogenesis. The murine *Gata2* mRNA level was also found to decrease very soon after the induction of adipocyte differentiation [36]. *Cebpb* and *Cebpd* are early transcription factors and so usually have the same profile during adipogenesis in mouse cell lines, with peaks in the early hours or days of differentiation, but then declining [37]. In our differentiation system, this type of profile was observed only for *CEBPB*. The expression of *PPARG* and *CEBPA* gradually increased over the subsequent days of differentiation, while the *FABP4* gene was upregulated in the final day of differentiation, which is in accordance with observations in other murine or porcine in vitro adipogenesis systems [35,38].

The analysis of the expression profiles of *SREBF1a* and *SREBF1c* in AD-MSC$_{WT}$ showed that an increase in the expression of *SREBF1a* takes place in the early stages of differentiation, which is why *SREBF1c* is upregulated in the late stages of adipogenesis. The same trend was observed in 3T3-F442A cells where the expression of *Srebf1a* was found in the very early stage of induction, before *Srebf1c* [23]. However, it should be pointed out that the 3T3-F442A cell line shows very rapid adipocyte differentiation in vitro, taking only hours with the use of staurosporine or dexamethasone induction [39]. The entire differentiation process lasted six days. Our study was conducted on MSC derived from adipose tissue, which reflects a more physiological process, as this type of MSC has a higher adipogenic

differentiation capacity [40]. Additionally, we also showed in this study for the first time the differences in the expression level of two isoforms of the *SREBF1* gene in subcutaneous and visceral adipose tissue in pigs: *SREBF1c* was the predominant isoform expressed in these porcine adipose tissues. This is in accordance with data on humans showing that this isoform has greater expression in different adipose tissue depots than does *SREBF1a* [41].

The use of the CRISPR/Cas9 editing method to generate a deletion in the *SREBF1* gene, so as to disrupt the *SREBF1c* isoform but not the *SREBF1a* isoform, allowed comprehensive analysis of the expression pattern of genes that encode the crucial transcriptional factors and marker proteins for adipogenesis in the absence of *SREBF1c*. This modification had no effect on *GATA2* gene expression in AD-MSC$_{DEL}$. This may be due to the fact that this factor is expressed only in the very early stages of adipogenesis (before expression of the *SREBF1c* isoform) and its downregulation is necessary to induce differentiation into adipocytes [31,36]. The *CEBPD* gene expression pattern was also similar in both AD-MSC$_{DEL}$ and AD-MSC$_{WT}$, which may indicate that there is no direct link between *CEBPD* and *SREBF1c* transcriptional factors. An interesting observation was made for the *CEBPB* gene, which was the only one to show a higher expression in AD-MSC$_{DEL}$ than in AD-MSC$_{WT}$. It is known that C/EBP transcription factors may regulate the SREBP1c gene expression during adipogenesis. For example, C/EBPβ can directly regulate SREBP1c by binding to its promoter region, and mice lacking C/EBPβ have reduced SREBP1c levels in adipose tissue [42]. It has also been shown that SREBP-1c can directly transactivate the C/EBPβ promoter [43]. Another study has shown that C/EBPβ phosphorylation promotes the expression of *Srebf1a*, which induces the expression of other adipogenic genes like *Pparg*, *Cebpa*, and *Srebf1c* [23]. So far, there have been no data on the impact of the lack of *SREBP1c* expression on *CEBPB* expression. It can be assumed that if the *SREBP1c* transcript is missing, a compensatory mechanism could lead to increased expression of the *CEBPB* gene. Our observation that *CEBPB* expression is increased in AD-MSCs lacking *SREBF1c* expression may suggests that *CEBPB* may respond to the loss of *SREBF1c* to maintain the adipogenic process, possibly helping to stabilize adipocyte development in the absence of this key isoform. Decreased expression was observed for the *CEBPA* gene, and complete inhibition of the expression of the *PPARG* and *FABP4* genes in AD-MSC$_{DEL}$ was noted. Since *SREBF1* expression is essential for providing lipid ligands to PPARγ [16], the disruption of *SREBF1c* transcription can block the expression of *PPARG* itself and its downstream target, *FABP4* [44]. Given the regulatory relationship between PPARγ and C/EBPα [19], the absence of *SREBF1c* may consequently lead to reduced *CEBPA* expression. The study conducted by the authors of [23] showed that the order of expression of adipogenic genes during adipogenesis using 3T3-F442A cells is as follows: *Cebpb*, *Srebf1a*, *Pparg2*, *Cebpa*, *Srebp1c*, and *Fabp4*. The expression pattern observed in our in vitro model of adipogenesis indicates that *CEBPA* is activated before *PRRAG* and *FABP4*. Since transcriptional regulation of adipogenesis is governed by the expression of the two main players *PPARG* and *CEBPA* [45], its absence blocks adipocyte differentiation; this was observed in our system as failure of lipid droplets to accumulate.

It is possible that the modification introduced in the *SREBF1c* gene locus may also affect the expression of other genes. A limitation of our study is its focus on a selection of genes specifically associated with adipogenesis and mature adipocytes. To address this, future research should employ RNA-Seq analysis, which would provide a comprehensive dataset and deeper insights into gene expression changes across the transcriptome.

Our studies have demonstrated the effectiveness of the CRISPR/Cas9 method as a gene-editing tool for examining gene functions. By generating MSCs with a deletion in the regulatory region of the *SREBF1* gene, we were able to identify the critical role of the *SREBF1c* isoform in adipogenesis. To the best of our knowledge, this is the first experiment of this type in the pig. To date, such experiments have been conducted only in other organisms. *Srebp*-1c knockout mice have been used to investigate the upstream and downstream regulatory mechanisms of SREBP-1c in vitro [46]. Knockout of the *SREBF1* gene by the introduction of a mutation (a 1bp deletion to produce a termination codon)

using CRISPR/Cas9 has previously been conducted for zebrafish [47], where it gave insight into the biology of *SREBF1* in the fatty metabolism and musculoskeletal functioning of the zebrafish. In our study, we introduced a mutation to this gene, in the form of a large deletion of the 5′ regulatory region. A similar approach has been used to introduce large deletions in the porcine *SRY* gene for study causes of sex reversal in gene-edited pigs [48] and a 93-bp deletion in the 3′-untranslated region (UTR) of the TNFα gene to generate a porcine Crohn's disease model [49]. This shows how advances in CRISPR/Cas9 technology have strongly impacted swine research, enabling precise genetic changes that could improve pig breeding, increase disease resistance, and develop biomedical models [50–52].

Modifying porcine MSCs with CRISPR/Cas9 technology shows promise in creating animal research models for human diseases, including obesity. This technology has been used to silence genes crucial for adipocyte differentiation, such as *PPARG* and *FKBP5* [53], as well as to target three genes (*SOCS3*, *DUSP1*, and *SIK1*) that are upregulated in the adipose tissue of patients with nonalcoholic fatty liver disease (NAFLD) [54]. These experiments were performed on preadipocytes derived from adipose tissue and human adipose-derived mesenchymal stem cells (hADMSCs), respectively. Researchers have also developed human brown-like cells (HUMBLE) by activating the *UCP1* gene in human white preadipocytes using CRISPR/Cas9 technology [55]. Additionally, there are examples of using ribonucleoproteins (RNPs) consisting of the Cas9 protein and sgRNA to disrupt the *NRIP1* gene in mouse and human progenitor cells ex vivo prior to their differentiation into adipocytes. This approach enabled the generation of adipocytes with beige characteristics [56].

Building on these technologies, it is expected that in the future, pigs could be genetically engineered to exhibit specific fat properties or optimized fat content in their meat, tailored to meet diverse applications or dietary preferences. A recent example of such a study is the creation of *LGALS12* knockout piglets using the CRISPR/Cas9 method combined with somatic cell nuclear transfer (SCNT) technology [57]. The *LGALS12* gene has been identified as important for porcine fat deposition. The study revealed that the absence of *LGALS12* suppresses preadipocyte proliferation and affects lipogenesis in porcine intramuscular and subcutaneous adipocytes. Further studies are needed to improve fat traits in pigs through genetic modifications of different loci.

4. Materials and Methods

4.1. Cell Culture and Induction of Adipogenesis

Mesenchymal stem cells isolated from adipose tissue (AD-MSC) were cultured in Advanced DMEM medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Sigma-Aldrich, Darmstadt, Germany), 5 ng/mL FGF-2 (PromoKine, Heidelberg, Germany), 2 mM L-Glutamine (Gibco), 1 mM 2-mercaptoethanol (Sigma-Aldrich), 1× antibiotic antimycotic solution (Sigma-Aldrich), and 1× MEM NEAA (Gibco) at 37 °C in 5% CO_2. After the cells reached confluency, adipogenesis was induced using a differentiation medium. The differentiation medium was composed of Advanced DMEM (Gibco), 10% FBS (Sigma-Adrich), 1× antibiotic antimycotic solution (Sigma-Aldrich), 5 ng/mL FGF-2 (PromoKine), 1× Linoleic Acid Albumin (Sigma-Aldrich), 1× ITS Supplement (Sigma-Aldrich), 1 µm Dexamethasone (Sigma-Aldrich), 100 µm Indomethacin (Sigma-Aldrich), and 50 µm IBMX (Sigma-Aldrich). Differentiation was allowed to occur over ten days, with the medium being changed every 24 h.

4.2. Monitoring of Lipid Droplet Formation

The accumulation of lipid droplets was monitored every day by visual examination under phase-contrast microscopy (TS100 Eclipse, Nikon, Melville, NY, USA). BODIPY staining was performed by fixing cells with 4% paraformaldehyde in PBS (*w*/*v*) for 10 min at room temperature and washed with PBS three times. The cells were then incubated with BODIPY (Life Technologies, Grand Island, NY, USA) in PBS (2.7 µg/mL) and washed three times in PBS. The nuclei were counterstained with DAPI in Vectashield medium (Vector Laboratories, Newark, CA, USA). The BODIPY fluorescence intensity was measured using

a Nikon E600 Eclipse microscope (Melville, NY, USA) and Lucia software version 1.0 (Laboratory Imaging, Prague, Czech Republic) and quantified using ImageJ software version 1.54g (NIH, Bethesda, MD, USA).

4.3. gRNA Design and MSC Transfection

gRNAs targeting the 5′-flanking region of the *SREBF1c* gene were designed using the CRISPOR tool (http://crispor.tefor.net/ accessed on 15 January 2022). The sequence of gRNA is shown in Table S1. The gRNA oligos with a BbsI overhang were cloned into the pX330 vector (Addgene #42230, Cambridge, MA, USA) carrying a U6 promoter, an sgRNA scaffold sequence, and the puromycin resistance gene. Then, 2 µg DNA from two plasmids was used for cotransfection into AD-MSCs by nucleofection with a P2 Primary Cell 4D-Nucleofector X Kit (Lonza, Basel, Switzerland) using a 4D Nucleofector Lonza system (Lonza). Transfection efficiency was determined using a control plasmid (pmaxGFP Vector, Lonza). After transfection with plasmids DNA, cells were plated on six-well plates and, after the next 24 h, they were selected using 2 µg/mL puromycin for 48 h. Cells were further cultured to obtain single-cell colonies.

4.4. PCR Genotyping of AD-MSC Cells

PCR reactions were performed to screen cell colonies for a deletion in the 5′-regulatory sequence of *SREBF1c*. First, genomic DNA was isolated from single MSC colonies using QuickExtract DNA Extraction Solution (Biosearch Technologies, Petaluma, CA, USA) and from wild-type MSCs using MasterPure Complete DNA & RNA Purification kit (Biosearch Technologies). PCR reactions were performed using *SREBF1c* gRNA1 F: 5′ctgagactgctggggagtgt and *SREBF1c* gRNA3 R: 5′tcaggagcgggctctcac primers. The expected PCR product was 954 bp in unmodified cells and approximately 400 bp in MSC colonies with a deletion in the 5′-regulatory sequence of *SREBF1c*.

4.5. Sanger DNA Sequencing

The PCR products were purified using Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Waltham, MA, USA). After the purification, a BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific) was used to generate fragments that were subsequently filtered using Sephadex G-50 (Sigma-Aldrich). Capillary electrophoresis was run on 3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), and the resulting chromatograms were analyzed using the SeqMan Pro (DNASTAR) software version 12.2.0 package.

4.6. RNA Isolation, cDNA Synthesis, and Real-Time PCR

Total RNA was extracted from AD-MSC cell cultures as well as from subcutaneous and visceral adipose tissues using TriPure Isolation Reagent (Roche Diagnostic, Mannheim, Germany). After quantitative and qualitative analyses with a Nanodrop 2000 (Thermo Scientific) spectrophotometer, 1 µg of RNA was reverse-transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR primers were designed for each transcript to anneal to different exons using the Sscrofa 11.1 reference sequence (Table S2). PCR reactions were performed in triplicate on a Light Cycler 480 II (Roche) instrument with a Light Cycler 480 SYBR Green I Master Kit (Roche). The relative mRNA levels of tested genes were quantified using the second derivative maximum method (Roche), and the results were normalized using the geometric mean of expression of *RPL27* and *PPIA* as reference genes [58,59].

4.7. Experimental Control Procedures

All results obtained from CRISPR/Cas9 editing were compared to unedited cells, which served as the control group. To verify the functionality of the CRISPR/Cas9 system, a fluorescent reporter system using a vector containing GFP was employed. In silico prediction tools were utilized to identify potential off-target sites, and only sgRNAs with

an extremely low probability of off-target modifications were selected. Two different techniques were used to validate the results: PCR with gel electrophoresis and Sanger sequencing. The experiments were conducted with multiple replicates (n = 3). Only cells obtained from single colonies in which the deletion was confirmed by PCR and Sanger sequencing were selected for the next steps of the study—differentiation into adipocytes. Appropriate statistical analyses were performed to evaluate the significance of the results.

4.8. Statistical Analysis

The analysis was conducted using IBM SPSS Statistics 28. A significance level of 0.05 was adopted. To compare the results of repeated measurements between the study groups, a MANOVA with repeated measures was used. Sphericity was assessed using Mauchly's test, and, in the case of non-sphericity, the Greenhouse–Geisser correction was applied to the MANOVA. Due to the small number of measurements, a detailed analysis of the descriptive statistics was also performed, as presented in the tables and graphs. When sphericity assumptions could not be met and MANOVA was not feasible, the t-test was used to compare the data between groups in successive measurements, while the Friedman test was used to compare the results within the same group across multiple measurements. The Shapiro–Wilk test was chosen to assess the normality of the distributions. To compare results between two groups, a non-parametric independent samples test (the Mann–Whitney U-test) was used due to the lack of normal distribution.

5. Conclusions

The use of the CRISPR/Cas9 method to generate modified porcine MSCs with a large deletion in the regulatory region of the *SREBF1* gene allowed us to determine the poorly-understood role of the *SREBF1c* isoform as an important factor in the process of adipogenesis in the pig. These findings not only contribute to a deeper understanding of the molecular mechanisms underlying the formation of fat cells in the pig but also open new possibilities for targeted genetic modifications to influence fat deposition in pigs. This could improve meat quality in the pig industry and support the development of porcine models for human obesity research.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ijms252312677/s1.

Author Contributions: Conceptualization: M.S., T.F. and I.S.; formal analysis: M.S., T.F. and I.S.; funding acquisition: I.S.; investigation: M.O.A., A.B., M.S., T.F. and I.S.; methodology: M.O.A., A.B., M.S., T.F. and I.S; project administration: I.S.; resources: I.S. and T.F.; supervision: I.S.; writing—original draft: M.O.A., M.S., T.F. and I.S.; writing—review and editing: M.O.A. and I.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financed by the Polish National Science Center, Grant No. 2018/29/B/NZ2/00956.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of the study are available from the corresponding author (I.S.) upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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Supplementary materials

Deciphering the role of the *SREBF1* gene in the transcriptional regulation of porcine adipogenesis using CRISPR/Cas9 editing

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Figure S1. GFP expression in AD-MSC after nucleofection with control plasmid.

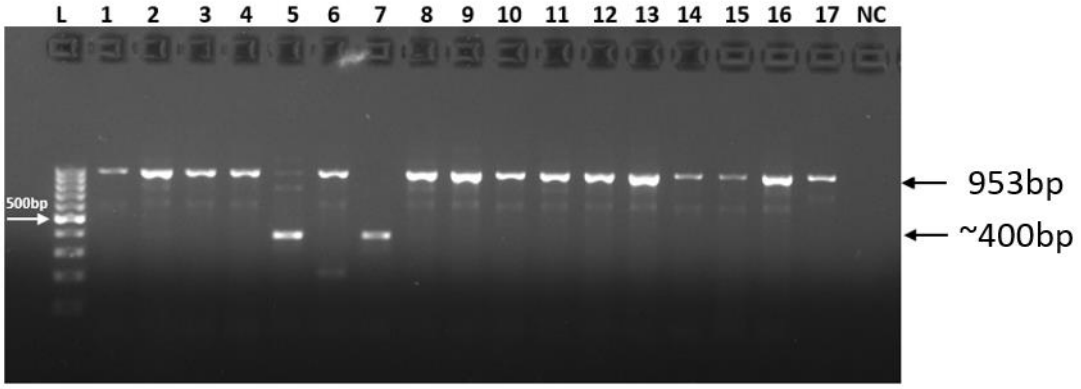

Figure S2. PCR detection of a deletion in the 5'-regulatory sequence of *SREBF1c* in single AD-MSC colonies. The expected PCR product size was 953 bp in unmodified MSC (WT), but shorter (~400 bp) in the targeted MSC colonies (lines 5 and 7). L: 100–1000 bp DNA ladder; NC: negative control (sample with no DNA template).

Figure S3. Relative mRNA levels of *SREBF1a* and *SREBF1c* isoforms in the subcutaneous and visceral adipose tissue of wild type pigs.

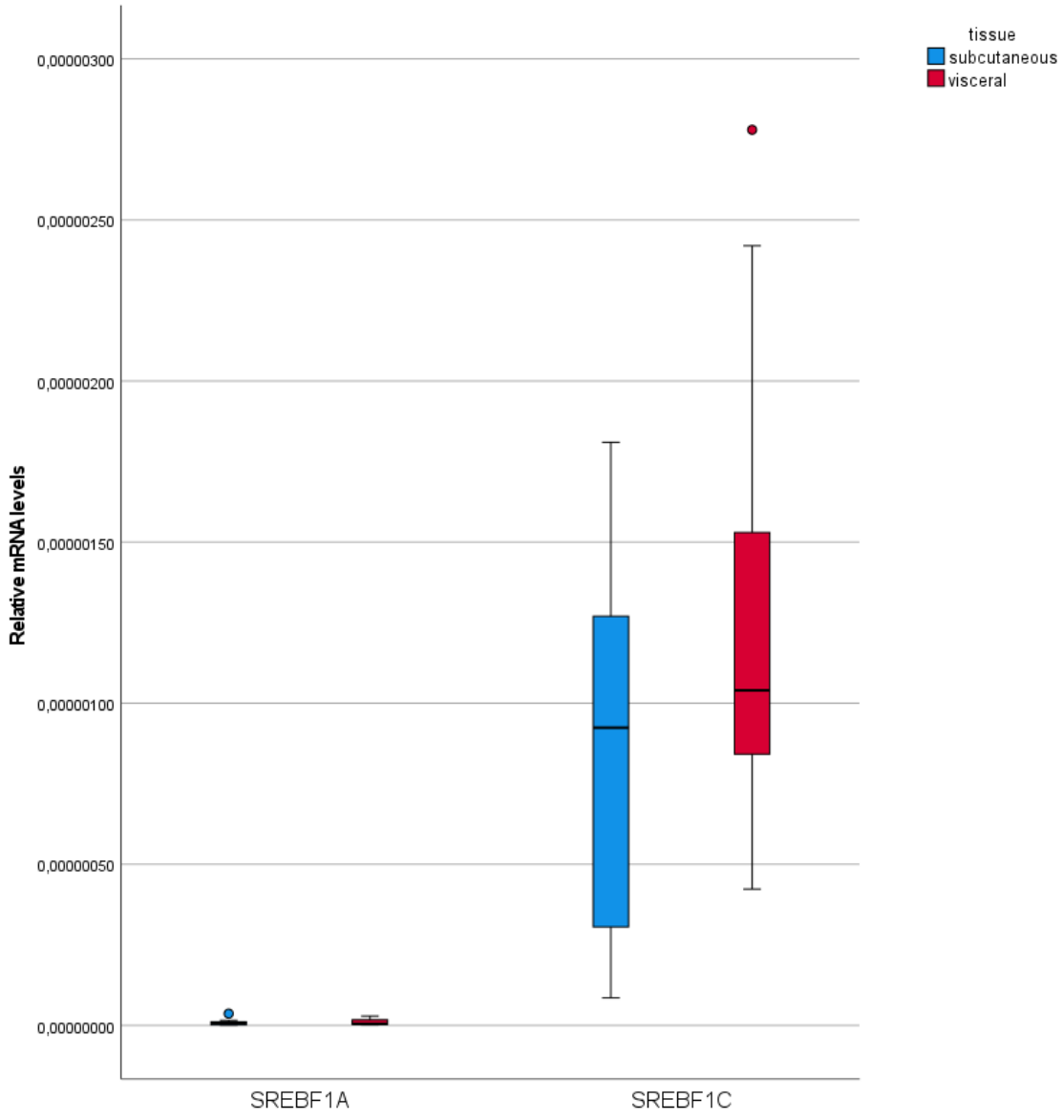

Table S1. gRNAs/primers directed against the 5'flanking region of *SREBF1c* gene.

gRNA	Name	Sequence
gRNA1	SREBF1c_g1_F	CACCGtacttgtccccgtcgatgca
	SREBF1c_g1_R	AAACtgcatcgacggggacaagtaC
gRNA3	SREBF1c_g3_F	CACCGaccatggactgcacgttcga
	SREBF1c_g3_R	AAACtcgaacgtgcagtccatggtC

Table S2. List of primer pairs used in RT-qPCR.

Gene	Primer sequence	Product size (bp)	Annealing temperature (°C)	Genbank accession number
*RPL27**	F: 5` GCAAAGCGGTCATCGTAAA R: 5` CTTGTGGGCATGAGGTGAT	190	60	NM_001097479.1
*PPIA**	F: 5 `CACAAACGGTTCCCAGTTTT R: 5 `TGTCCACAGTCAGCAATGGT	171	60	XM_021078519.1
PPARG	F: 5` GCATCAGCTCTGTGGACCTG R: 5` GATCAGCTCTCGGGAATGGG	132	60	XM_005669783
CEBPA	F: 5` CGTGAGCGCAACAACATCG R: 5` CTCAGTTGTTCCACCCGCTT	131	60	NC_010448
FABP4	F: 5` TTCAAATTGGGCCAGGAAT R: 5` ATTCTGGTAGCCGTGACACC	191	60	NM_001002817
CEBPD	F: 5` TGGTTGCTGTTGAAGAGGTCA R: 5` CCATCGACTTCAGCGCCTAC	97	60	XM_005663091.2
CEBPB	F: 5` TACTACGAGGCGGACTGCTT R: 5` TCCAGGTATGGGCTGAAGTC	152	60	NM_001199889.1
GATA2	F: 5` CTCCAGCTTCACCCCTAAG R: 5` CCCGTTCATCTTGTGGTACAG	157	60	XM_021068525.1
SREBF1c	F: 5` TCGAAGACATGCTTCAGCTC R: 5` GGAGCTCATGGTGGAAGGAG	155	60	XM_021066226.1
SREBF1a	F: 5` ACAGAGGCAGCCTTGGAG R: 5` GTCGCTGTCTTGGTTGTTGA	108	60	NM_214157.1

* *reference gene*

Journal of Applied Genetics (2026) 67:477–486
https://doi.org/10.1007/s13353-025-01030-x

ANIMAL GENETICS • ORIGINAL PAPER

The role of the *CEBPB* gene in porcine adipogenesis: a study using CRISPR/Cas9-edited mesenchymal stem cells

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Received: 14 July 2025 / Revised: 16 October 2025 / Accepted: 14 November 2025 / Published online: 26 November 2025

Abstract

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 is a powerful tool for gene editing and the regulation of gene expression. It enables the introduction of targeted mutations, thereby facilitating functional studies of specific genes in various cellular processes. In this study, we aimed to generate a deletion in the promoter region of the *CEBPB* gene, which encodes a transcription factor involved in adipogenesis, and to evaluate the impact of this modification on the adipogenic differentiation potential of porcine mesenchymal stem cells (MSCs). A 575-bp deletion was introduced in the target region, resulting in the generation of both homozygous and heterozygous mutant cells. Adipogenic differentiation was assessed by quantifying transcript levels of adipocyte marker genes (*GATA2*, *CEBPA*, *PPARG*, and *FABP4*) at days 0, 4, 6, 8, and 10 of the differentiation process. Disruption of *CEBPB* expression led to the downregulation of these adipogenic markers, indicating impaired adipocyte differentiation. Additionally, to assess the proliferative capacity of the edited cells, the expression levels of proliferation-associated genes (*CCND1*, *MCM2*, and *PCNA*) were measured. A reduction in their transcript levels was observed in both homozygous and heterozygous mutant cells. These findings indicate that both homozygous and heterozygous deletions in the *CEBPB* promoter completely block adipogenesis and alter MSC proliferation, highlighting the pivotal role of *CEBPB* not only in adipogenic differentiation but also in the regulation of cell proliferation in porcine mesenchymal stem cells. These results provide new insights into the molecular mechanisms underlying adipose tissue development and have implications for pig breeding strategies aimed at optimizing carcass composition, as well as for biomedical research focused on adipose tissue biology.

Keywords Adipogenesis · CEBPB · CRISPR/Cas9 · MSC · Pig · Proliferation

Introduction

Adipogenesis—the process of fat cell development—is of particular interest in pig research, as adipose tissue significantly influences growth rates, energy balance, and survival during early life, as well as traits relevant to meat quality and agricultural productivity. Furthermore, pigs are widely recognized as valuable biomedical models for human diseases, including obesity (Louveau et al. 2016; Stachowiak et al. 2016). Adipogenesis is regulated by a complex network of transcription factors that either promote or inhibit adipocyte differentiation. Among these, peroxisome proliferator-activated receptor gamma (PPARγ) and members of the CCAAT/enhancer-binding protein (C/EBP) family play central roles in orchestrating this process (Rosen and MacDougald 2006; Farmer 2006).

One of the transcription factors in the C/EBP family is CCAAT/enhancer-binding protein beta (C/EBPβ), which contains a basic leucine zipper (bZIP) domain and acts as an early regulator of adipogenesis (Guo et al. 2015). C/EBPβ activates the expression of two master regulators, C/EBPα and PPARγ, which in turn drive the expression of a wide array of adipocyte-specific genes responsible for lipid metabolism, insulin sensitivity, and other adipocyte functions. The *CEBPB* gene, which encodes C/EBPβ, is intron-free and broadly expressed across various cell types, where it contributes to cell differentiation, proliferation, and the

Communicated by: Tomasz Strabel

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regulation of immune and inflammatory responses (Pal et al. 2009; Ren et al. 2023).

The role of C/EBPβ in adipogenesis has been extensively studied in rodent and human models. For example, RNA interference-mediated knockdown of *Cebpb* in murine 3T3-L1 cells inhibits adipogenesis, and *Cebpb* knockout mice exhibit reduced fat accumulation (Zhang et al. 2011; Tanaka et al. 1997). Conversely, overexpression of C/EBPβ induces adipocyte differentiation in 3T3-L1 cells even in the absence of hormonal inducers (Yeh et al. 1995). C/EBPβ has also been shown to be essential for mitotic clonal expansion (MCE), a phase during which growth-arrested preadipocytes re-enter the cell cycle and undergo several rounds of division prior to differentiation (Tang et al. 2003). Although MCE appears to be required for murine adipogenesis, some studies suggest it may not be essential (Qiu et al. 2001), and it is reportedly dispensable in human adipocytes (Marquez et al. 2017).

Despite its well-documented role in other species, the regulatory function of C/EBPβ during porcine adipogenesis remains poorly understood. In an in vitro model using mesenchymal stem cells (MSCs) derived from porcine bone marrow, *CEBPB* expression was shown to be upregulated during the early stages of adipogenic differentiation and downregulated at later stages (Szczerbal et al. 2009). Similarly, studies in adipose-derived MSCs (AD-MSCs) demonstrated that *CEBPB* exhibits a peak of transcriptional activity on day 4 of differentiation (Aksoy et al. 2024). Interestingly, *CEBPB* was the only gene upregulated in AD-MSCs with a disrupted *SREBF1c* gene, suggesting a functional interaction between *CEBPB* and *SREBF1c* (Aksoy et al. 2024). Reciprocal regulation between C/EBPβ and SREBP-1c has also been demonstrated, with each capable of activating the other's promoter (Le Lay et al. 2002; Payne et al. 2009). Other studies have shown that the downregulation of the C/EBP family, including *CEBPB*, in porcine intramuscular preadipocytes leads to decreased *PPARG* expression, and the interaction between *CEBPB* and *FGF21* appears to play a significant role in adipogenesis (Wang et al. 2015). Furthermore, in a recent transcriptomic study comparing synovial MSCs from two pig breeds differing in growth and fat deposition, *CEBPB* was among the genes upregulated during the early stages of differentiation, when cells are actively proliferating (Ponsuksili et al. 2024).

Recent advances in CRISPR-based technologies have revolutionized the precision engineering of various genomic elements, including both coding and regulatory sequences. The ability to introduce targeted promoter deletions and to monitor cellular responses during key biological processes, such as adipogenesis, provides a powerful framework for elucidating gene function, which is of major importance for both biomedical and agricultural applications (Li et al. 2023; Fu et al. 2024). Novel approaches such as CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa), which employ catalytically inactive Cas9 (dCas9) to repress or activate gene expression, open new directions in stem cell engineering (Razavi et al. 2024a). Furthermore, the integration of CRISPR systems with microfluidic-based biosensors has enabled the creation of robust platforms for highly sensitive early diagnosis, precise disease monitoring, and real-time genetic analysis (Razavi et al. 2024b, c).

In the present study, we aimed to further elucidate the transcriptional regulatory role of *CEBPB* during porcine adipogenesis. We employed the CRISPR/Cas9 genome editing system to generate targeted deletions in the promoter region of the *CEBPB* gene in porcine MSCs. The creation of both homozygous and heterozygous knockout variants enabled us to investigate how *CEBPB* disruption affects the proliferation and differentiation potential of MSCs into adipocytes, thereby providing new insights into its functional role in porcine adipose tissue development.

Material and methods

Cell culture and induction of adipogenesis

Adipose tissue-derived mesenchymal stem cells (MSCs) were cultured in Advanced DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 5 ng/mL fibroblast growth factor-2 (FGF-2; PromoKine), 2 mM L-glutamine (Gibco), 1 mM 2-mercaptoethanol (Sigma-Aldrich), 1× antibiotic-antimycotic solution (Sigma-Aldrich), and 1× MEM non-essential amino acids (NEAA; Gibco). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Upon reaching confluence, adipogenic differentiation was induced by replacing the growth medium with differentiation medium composed of Advanced DMEM (Gibco), 10% FBS (Sigma-Aldrich), 1× antibiotic-antimycotic solution (Sigma-Aldrich), 5 ng/mL FGF-2 (PromoKine), 1× linoleic acid–albumin solution (Sigma-Aldrich), 1× insulin–transferrin–selenium (ITS) supplement (Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich), 100 µM indomethacin (Sigma-Aldrich), and 50 µM isobutylmethylxanthine (IBMX; Sigma-Aldrich). Differentiation was carried out over a period of 10 days, with the medium replenished daily. The accumulation of lipid droplets was monitored by visual examination under phase-contrast microscopy (TS100 Eclipse, Nikon, Melville, NY, USA) and BODIPY staining according to procedure described by Aksoy et al. (2024).

gRNA design and MSC transfection

Guide RNAs (gRNAs) targeting the promoter region upstream of the *CEBPB* gene were designed using the

CRISPOR tool (http://crispor.tefor.net/). The gRNA sequences are listed in Table S1. Each gRNA oligonucleotide pair was cloned separately into the pX330 SpCas9 with PuroR vector (based on Addgene plasmid #4223, kindly provided by Dr. T. Flisikowska from Chair of Livestock Biotechnology, TUM, Freising, Germany), which contains a U6 promoter to drive gRNA expression, an sgRNA scaffold, and a puromycin resistance gene for selection. A total of 2 µg of plasmid DNA, derived from two gRNA-containing vectors, was co-transfected into MSCs using the P2 Primary Cell 4D-Nucleofector™ X Kit (Lonza) and the 4D-Nucleofector™ System (Lonza). To assess transfection efficiency, a control plasmid encoding GFP (pmaxGFP Vector; Lonza) was used. Following transfection, cells were seeded in six-well plates and incubated for 24 h. Puromycin selection (2 µg/mL) was then applied for 48 h to enrich for transfected cells. After selection, surviving cells were cultured further to allow for the formation of single-cell colonies.

PCR genotyping to detect deletions in the 5′ regulatory region of *CEBPB*

To verify the introduction of deletions in the 5′ regulatory sequence of the *CEBPB* gene, PCR-based genotyping was performed on single-cell-derived MSC colonies. Genomic DNA was extracted from both transfected and wild-type (WT) MSCs using the MasterPure Complete DNA & RNA Purification Kit (Biosearch Technologies), according to the manufacturer's instructions. Primers for genotyping were designed using Primer3Plus software (sequences provided in Table S2). PCR amplification of the wild-type allele yielded a product of 818 base pairs (bp), whereas a 243-bp shorter amplicon was observed in colonies carrying the deletion within the 5′ regulatory region of *CEBPB*.

Sanger DNA sequencing

PCR products were purified using Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific), following the manufacturer's protocol. Sequencing reactions were carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The resulting sequencing products were purified using Sephadex G-50 columns (Sigma-Aldrich) prior to capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems). The resulting chromatograms were analyzed using the SeqMan Pro software (DNASTAR) and aligned against the porcine *CEBPB* reference sequence (ENSSSCG00000034207, https://www.ensembl.org/).

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was extracted from MSCs using TriPure Isolation Reagent (Roche), following the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). One microgram of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR primers were designed to span exon-exon junctions based on the Sscrofa11.1 reference genome (primer sequences are listed in Table S3). Quantitative PCR reactions were performed in triplicate using the LightCycler 480 II system (Roche) and the LightCycler 480 SYBR Green I Master Kit (Roche). Relative mRNA levels were calculated using the second derivative maximum method (Roche). The *RPL27* gene was used as the internal reference, and normalization was performed using the mean relative expression values of biological replicates.

Statistical analysis

The statistical analysis was conducted using IBM SPSS Statistics 28 software, with a significance level set at 0.05. To examine changes between two groups over consecutive days, a repeated measures MANOVA test was employed. Additionally, Pillai's trace and partial eta squared were calculated to evaluate the study's effect sizes. To assess differences in quantitative data between groups, a non-parametric test was applied due to the very small number of measurements. Specifically, the Wilcoxon test with the Holm-Bonferroni correction was used, as it is recommended for extremely small groups.

Results

Characterization of the 5′ regulatory region of the porcine *CEBPB* gene

An in silico analysis of the 5′ regulatory sequence of the porcine *CEBPB* gene was conducted to identify putative promoter elements and transcription factor binding sites associated with adipogenesis. The analysis was performed using the DNASTAR Lasergene software suite (https://www.dnastar.com/software/). Multiple regulatory motifs were identified, including a TATA box, CRE-like elements, glucocorticoid response elements (GRE), a coding sequence start (CDS), and the transcription start site (TSS) region (Fig. 1a). To excise this regulatory element-rich region, two out of four designed single guide RNAs (gRNAs) were

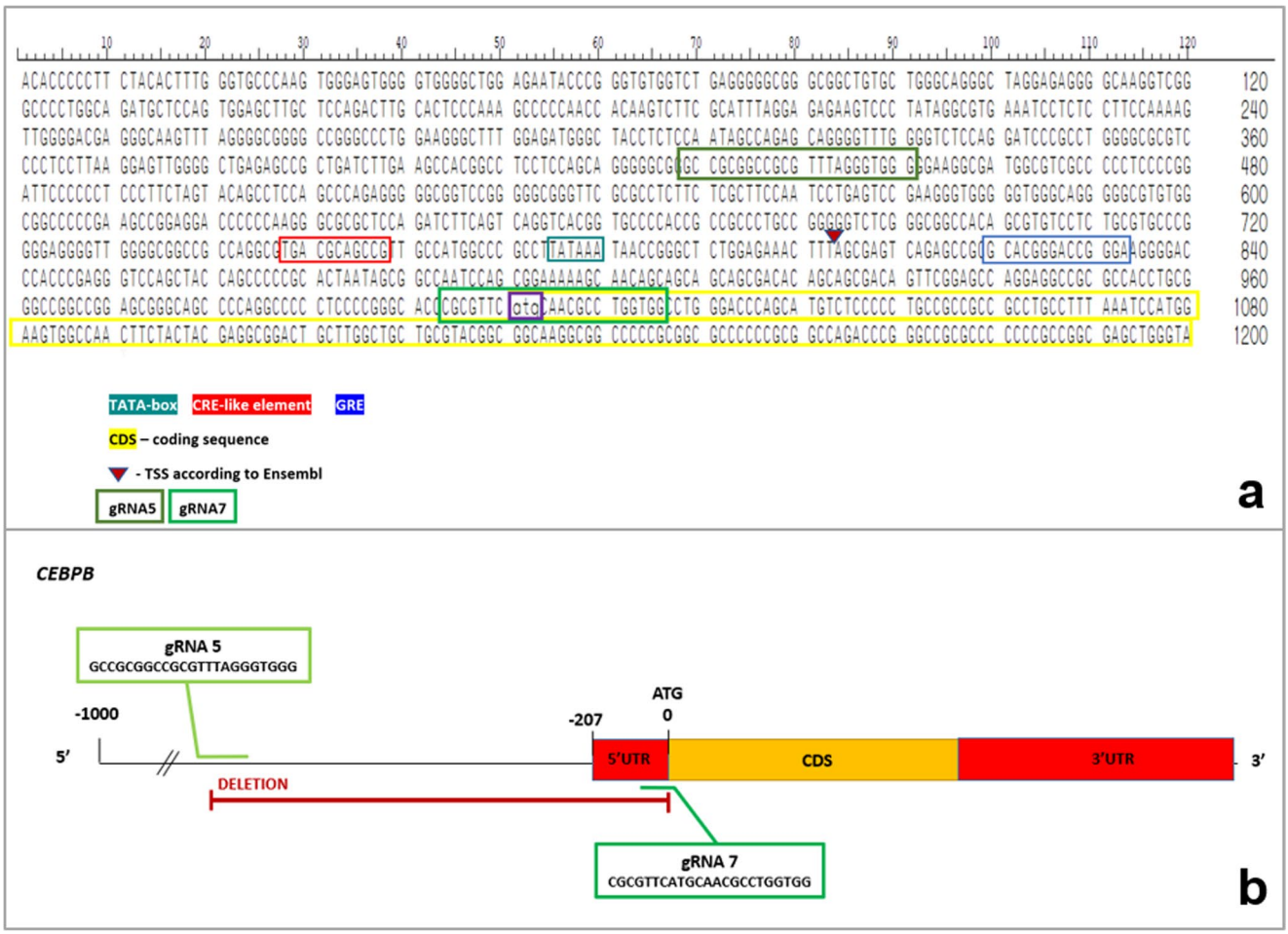

Fig. 1 Schematic representation of the 5′ regulatory region of the *CEBPB* gene showing predicted transcription factor binding sites relevant to adipogenesis, including TATA box, CRE-like elements, GRE, CDS, and TSS. The positions of gRNA5 and gRNA7 used to delete the promoter region are indicated (**a**). Genomic structure of the porcine *CEBPB* gene with the locations of the designed gRNAs used for CRISPR/Cas9-mediated editing (**b**)

selected for CRISPR/Cas9 targeting, resulting in the generation of two cleavage sites flanking the region of interest (Fig. 1b; Table S1).

Introduction of a deletion in the *CEBPB* locus

CRISPR/Cas9-mediated genome editing was employed to introduce a targeted deletion within the *CEBPB* locus. Guide RNAs were cloned into the pX330 plasmid vector, and MSCs were transfected via nucleofection. Transfection efficiency was determined using the pmaxGFP control plasmid (Lonza, Basel, Switzerland) by counting GFP-positive MSCs in randomly selected microscopic fields. The mean efficiency was estimated at approximately 60% (Fig. S1). Following transfection, single MSC colonies were genotyped by PCR. Primers were designed to amplify genomic DNA regions flanking the gRNA target sites. In wild-type cells, the expected PCR product was 818 base pairs (bp), whereas cells carrying the intended deletion yielded a 243-bp fragment, indicating a 575-bp deletion in the *CEBPB* promoter region (Fig. 2a). Based on this genotyping approach, a total of eleven clones with targeted deletions were identified—one homozygous and ten heterozygous. The editing efficiency at the *CEBPB* locus reached 22.9%, as determined by the proportion of edited clones ($n=11$) among all screened samples ($n=48$). Sanger sequencing of PCR amplicons confirmed the presence of a 575-bp deletion within the 5′ regulatory region of the *CEBPB* gene (Fig. 2b). No additional mutations, insertions or deletions (indels), or partial deletions were detected apart from the intended 575-bp deletion in the target region.

Expression profile of the *CEBPB* gene in wide-type MSC (MSC_{WT}) and in MSC with deletion in promoter region (MSC_{DEL})

To assess whether the deletion introduced in the promoter region of the *CEBPB* gene affected its transcriptional

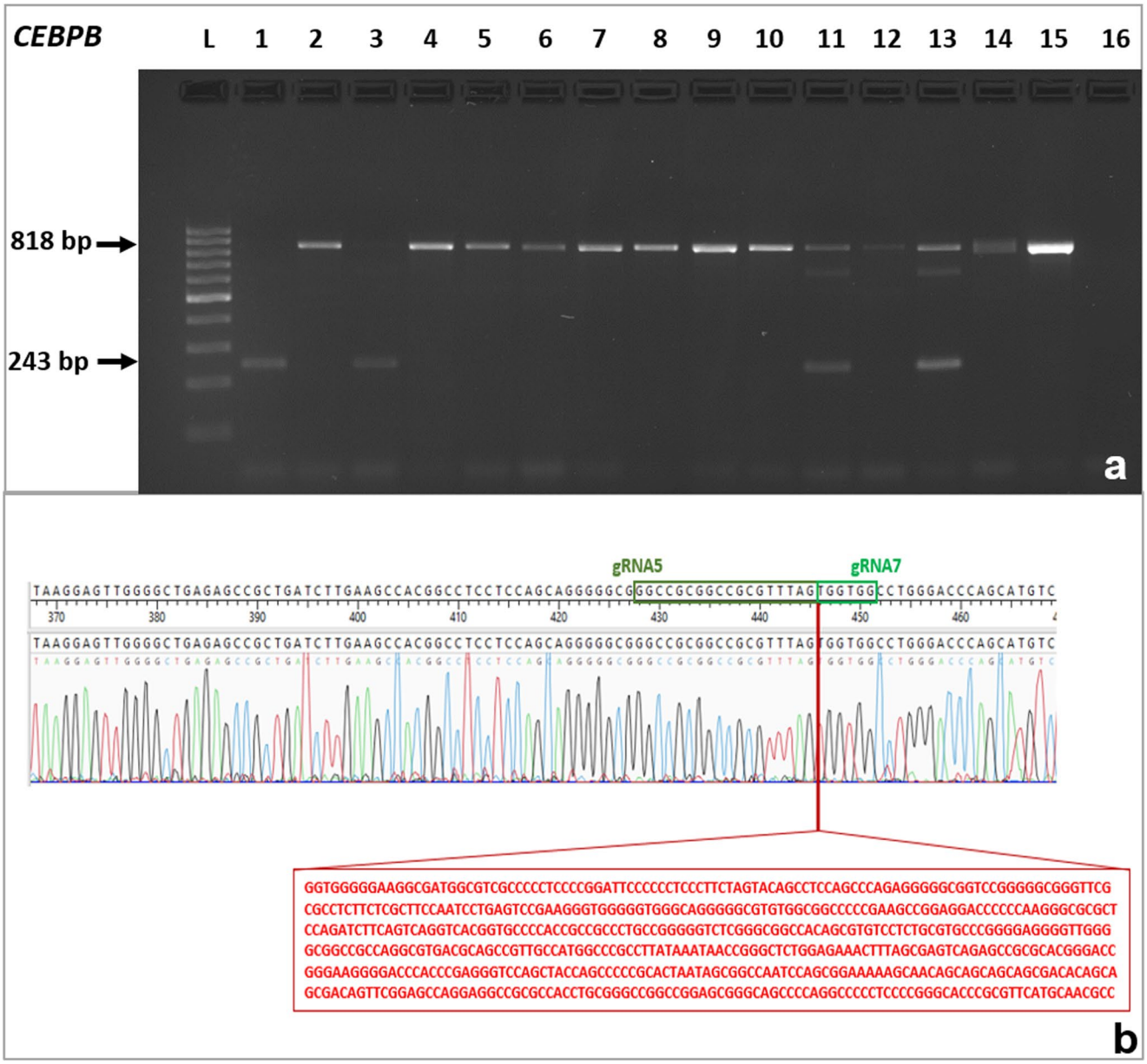

Fig. 2 Representative electrophoresis image of PCR genotyping from single-cell-derived colonies, showing detection of the *CEBPB* promoter deletion. L – 100 bp DNA ladder (Thermo Fisher Scientific); Lanes 2, 4–10, 12, 14 – wild-type colony (818 bp); lane 1 – colony with a homozygous deletion (243 bp); lanes 3,11,13 – colonies with a heterozygous deletion; lane 15 - negative control (non-transfected cells); lane 16 - negative control (no DNA template) (a). Sanger sequencing chromatogram confirming a 575 bp deletion in the 5′ regulatory region of the *CEBPB* gene. The sequence shows the junction between the excised DNA ends, with partial gRNA5 and gRNA7 recognition sequences indicated (b)

activity, relative mRNA expression levels were compared between wild-type cells (MSC_{WT}) and genetically modified cells (MSC_{DEL}) carrying either homozygous or heterozygous deletions. A significant overall difference in *CEBPB* transcript levels was observed among cell types (Wilcoxon signed-rank test, $p=0.004$). Post hoc analysis revealed that MSC_{WT} exhibited significantly higher *CEBPB* expression than MSC_{DEL} with heterozygous deletions ($p=0.025$) and homozygous deletions ($p=0.025$) (Fig. 3a). *CEBPB* expression in wild-type cells was approximately 11-fold higher than in heterozygous cells. Due to the markedly reduced proliferation of MSCs carrying a homozygous deletion, these cells could not be used for adipogenic differentiation analysis. Therefore, further differentiation studies were conducted only on MSC_{DEL} cells with heterozygous deletions, in comparison to MSC_{WT}. The expression profile of *CEBPB* was evaluated over the course of adipogenic differentiation, from day 0 (undifferentiated MSCs) to day 10. In

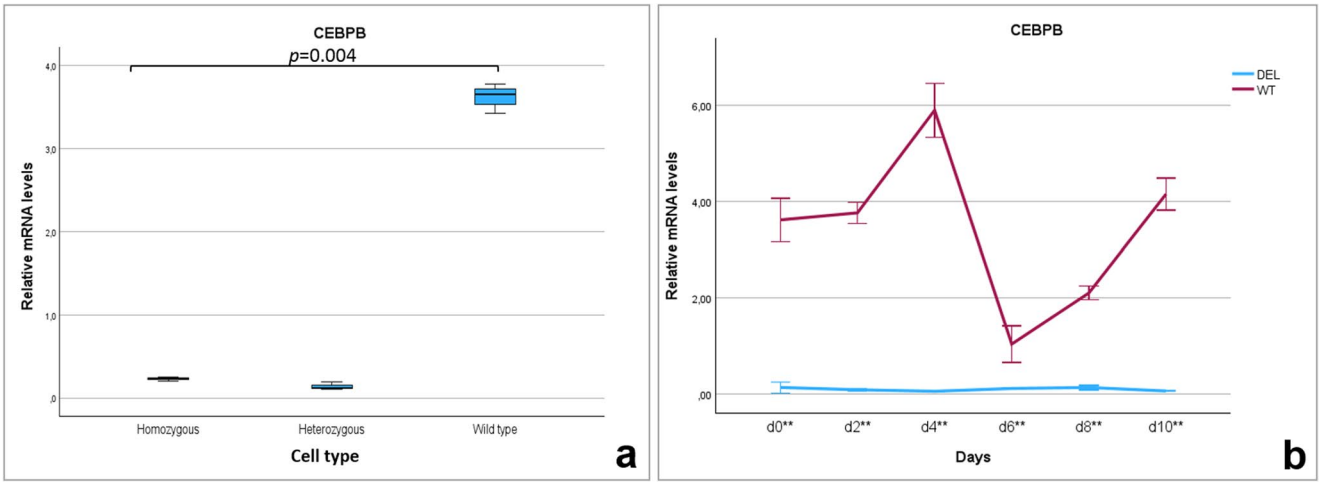

Fig. 3 Relative transcript levels of *CEBPB* gene in wide-type MSC (MSC_{WT}) and in MSC with deletion in promoter region (MSC_{DEL}) in homozygous and heterozygous cells, Wilcoxon test, $p<0.004$ (a) and during subsequent days of adipogenesis for heterozygous cells (b). The error bars represent 95% confidence intervals. **: significantly higher in MSC_{WT} than in MSC_{DEL}, MANOVA test, $p<0.001$

MSC_{WT}, *CEBPB* exhibited a characteristic peak in transcript levels on day 4 of differentiation. In contrast, MSC_{DEL} cells showed significantly reduced *CEBPB* expression across all examined time points (MANOVA, $p<0.001$) (Fig. 3b). These results demonstrate that the targeted promoter deletion effectively disrupted *CEBPB* expression, confirming the functional importance of this regulatory region during adipogenic differentiation.

Expression patterns of adipogenesis-related genes in MSC_{WT} and MSC_{DEL}

To assess the functional role of *CEBPB* in porcine adipogenesis, the expression profiles of key adipogenic marker genes were analyzed over the course of differentiation in MSC_{WT} and MSC_{DEL} (heterozygous) cells. In MSC_{WT}, the highest expression level of *GATA2* was observed at day 0, followed by a gradual decrease throughout the differentiation process. In contrast, MSC_{DEL}exhibited significantly lower *GATA2* expression compared to MSC_{WT} at all time points except day 10 (MANOVA, $p<0.001$) (Fig. 4a). *CEBPA* expression in MSC_{WT} progressively increased during adipogenesis, reaching its peak at day 10. In MSC_{DEL}, *CEBPA* transcript levels were significantly lower than those in MSC_{WT} across all days of differentiation (MANOVA, $p<0.001$) (Fig. 4b). A significant induction of *PPARG* expression was detected in MSC_{WT} starting from day 6. In MSC_{DEL}, *PPARG* expression was significantly reduced relative to MSC_{WT} at all time points, except on day 6 (MANOVA, $p<0.001$) (Fig. 4c). The expression of *FABP4*, a marker of mature adipocytes, was also altered. MSC_{DEL} exhibited significantly lower *FABP4* transcript levels than MSC_{WT} at days 0, 6, 8, and 10, while significantly higher levels were observed at days 2 and 4 (MANOVA, $p<0.001$) (Fig. 4d).

These results demonstrate that disruption of *CEBPB* expression interferes with the normal transcriptional cascade of adipogenesis, suppressing the expression of upstream transcription factors (*GATA2*, *CEBPA*, *PPARG*) as well as *FABP4*, a gene characteristic of terminal adipocyte differentiation. The downregulation of these markers corresponded with the observed phenotypic effect—cells with the *CEBPB* promoter deletion failed to accumulate lipid droplets, indicating an absence of adipogenic differentiation at the cellular level (Fig. S2).

Assessment of the proliferative status of MSC_{WT} and MSC_{DEL}

Due to the observed reduction in proliferation in *CEBPB*-modified cells, additional analyses were conducted to assess the expression of three proliferation-associated marker genes: *CCND1*, *MCM2*, and *PCNA*. A significant decrease in transcript levels of all three genes was detected in MSC_{DEL} cells —both heterozygous and homozygous—compared to unmodified MSC_{WT} cells (Wilcoxon signed-rank test, $p=0.004$) (Fig. 5). These results indicate that *CEBPB* plays a dual role in porcine mesenchymal stem cells, being essential not only for adipogenic differentiation but also for the regulation of cell proliferation.

Discussion

A deeper understanding of the molecular mechanisms regulating genes essential for adipose tissue development is crucial not only for advancing obesity treatment strategies in humans but also for improving the nutritional and health-promoting qualities of meat in livestock through controlled

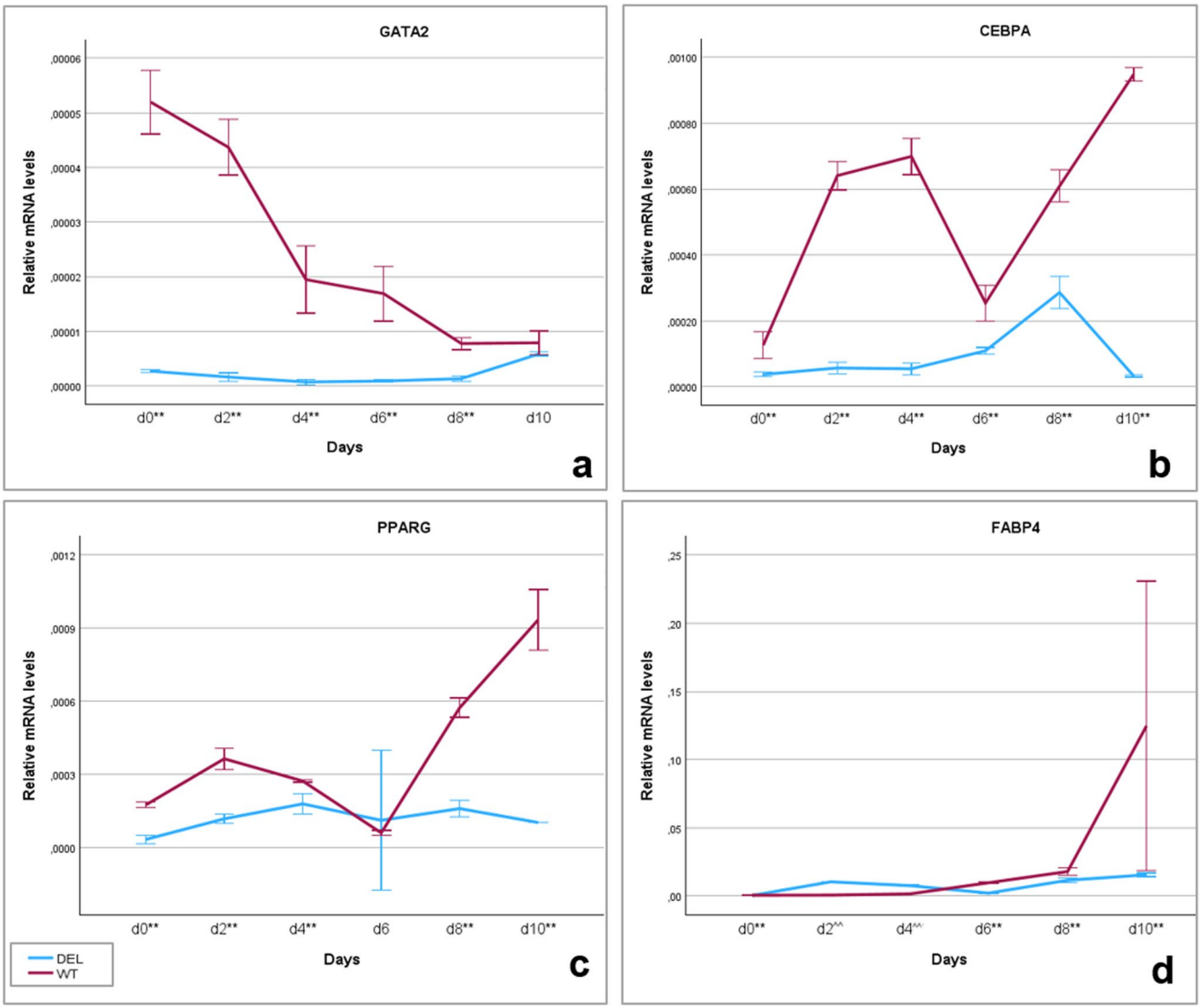

Fig. 4 Relative transcript levels of *GATA2* (**a**), *CEBPA* (**b**), *PPARG* (**c**) and *FABP4* (**d**) during subsequent days of adipogenesis in MSC_{WT} and MSC_{DEL}. Error bars represent 95% confidence intervals. **: significantly higher in MSC_{WT} than in MSC_{DEL}, $p<0.001$; ^^: significantly lower in MSC_{WT} than in MSC_{DEL}, MANOVA test, $p<0.001$

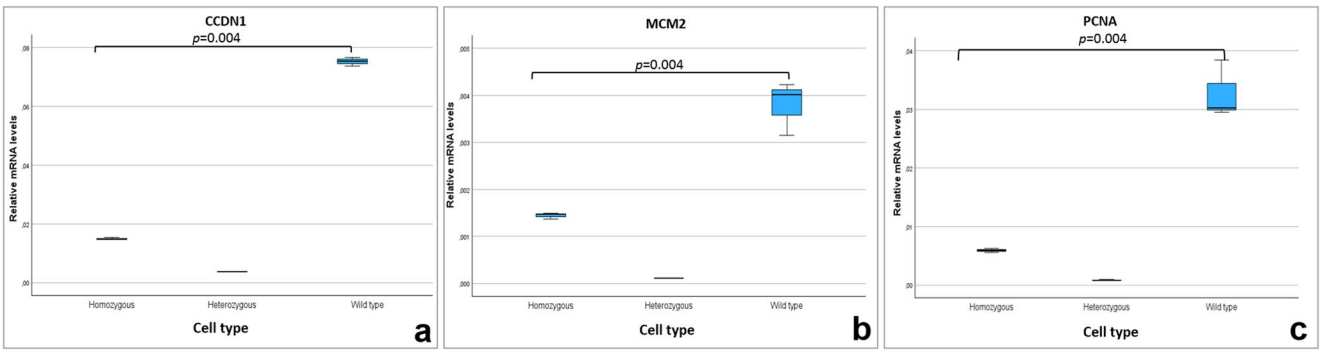

Fig. 5 Relative transcript levels of *CCDN1* (a), *MCM2* (b) and *PCNA* (c) genes in wide-type MSC (MSC_{WT}) and in MSC with deletion in promoter region of *CEBPB* gene (MSC_{DEL}) in homozygous and heterozygous status, Wilcoxon test, $p<0.004$

fat deposition. Genome editing technologies, particularly CRISPR/Cas9, have been successfully applied in both human and rodent models, including adipose-derived mesenchymal stem cells (MSCs) (Khademi et al. 2024). Targeted genes in these models have been implicated in diverse biological processes such as adipogenesis, lipid metabolism, thermogenesis, and adipocyte browning (Kamble et al. 2020; Lopez-Yus et al. 2023; Karami et al. 2025). Pigs have been used as large-animal models for CRISPR/Cas9-mediated gene editing to study genes related to fat accumulation including *UCP1* (Tsagkaraki et al. 2021), *LGALS12* (Wu et al. 2024), and *SREBF1* (Aksoy et al. 2024).

In the present study, we selected the *CEBPB* gene, which encodes an early-acting transcription factor involved in adipogenesis, for CRISPR/Cas9-mediated disruption. Initially, we hypothesized that since *CEBPB* acts upstream of master regulators such as *PPARG* and *CEBPA*, its silencing would reduce—but not completely abolish—the adipogenic potential of MSCs. However, our results revealed that both homozygous and heterozygous deletions in the *CEBPB* promoter led to complete inhibition of adipogenesis in porcine MSCs. These findings are consistent with recent results from a study applying CRISPR interference (CRISPRi) in human preadipocytes, where *CEBPB* knockdown also blocked adipocyte differentiation. In that study, the authors observed reduced expression of adipogenic markers at days 0 and 8 of differentiation, including a > 60% reduction in *FABP4* expression at day 8 (Bielczyk-Maczyńska et al., 2023). The marked decrease in *CEBPB* transcript levels observed in both homozygous and heterozygous cells indicates that the reduction is primarily caused by the loss of promoter activity. However, our current data do not exclude potential compensatory mechanisms or indirect effects involving distal regulatory elements or chromatin architecture. Further analyses, including chromatin accessibility profiling and transcriptional activity assays, will be required to determine whether additional regulatory pathways contribute to the observed downregulation of *CEBPB*.

The complete inhibition of adipogenesis observed even in heterozygous *CEBPB* promoter-deletion clones suggests a strong dosage sensitivity of *CEBPB* expression during the early stages of adipocyte differentiation. This phenomenon may reflect haploinsufficiency, where a single functional allele is insufficient to maintain the threshold level of transcription factor activity required to initiate the adipogenic cascade. Although *CEBPB* is not typically classified as an allelic imbalance gene, as reported for other loci involved in adipogenesis and lipid metabolism in pigs (Stachowiak et al. 2018), its pronounced dosage dependence supports the notion that partial loss of promoter function can lead to a functionally haploinsufficient state. The data therefore indicate that adipogenesis in porcine mesenchymal stem cells is highly dependent on precise quantitative control of *CEBPB* expression.

In addition to its role in differentiation, our study demonstrates that *CEBPB* is also involved in regulating MSC proliferation. Cells with *CEBPB* promoter deletions exhibited significantly reduced expression of proliferation-associated genes (*CCND1*, *MCM2*, and *PCNA*), and those with homozygous deletions showed poor in vitro growth. While no prior studies have examined how CRISPR/Cas9-mediated *CEBPB* knockout affects MSC proliferation, data from CRISPRi experiments in preadipocytes suggest that *CEBPB* silencing alters early gene expression programs. Bielczyk-Maczyńska et al. (2023) found that the highest number of differentially expressed (DE) genes occurred at days 0 and 4 of differentiation following *CEBPB* knockdown. Although cell proliferation-related pathways were not explicitly enriched in their gene set enrichment analysis (GSEA), the analysis was limited to 10 hallmark gene sets. Moreover, studies in cancer biology have demonstrated that cells with *CEBPB* knockdown have impair cell proliferation (Wang et al. 2023; Tan et al. 2025), supporting our observation that this gene also influences proliferative capacity in porcine MSCs.

Given the pivotal role of *CEBPB* in initiating the adipogenic transcriptional cascade, targeting such an essential regulator with CRISPR/Cas9 results in complete blockade of differentiation and reduced proliferative capacity, making it difficult to investigate downstream regulatory mechanisms. Therefore, future genome editing experiments aimed at dissecting the transcriptional control of adipogenesis may benefit from focusing on genes with secondary or modulatory roles, whose disruption does not fully abrogate the differentiation process but rather alters specific pathways, such as lipid metabolism or energy homeostasis. For instance, Lopez-Yus et al. (2023) used CRISPR/Cas9 to knock down *SOCS3*, *SIK1*, and *DUSP1* in human adipose-derived MSCs. Although these genes were not essential for adipocyte formation, their silencing altered lipid accumulation and metabolic gene expression profiles, allowing a more nuanced interpretation of their functions without completely blocking adipogenesis. Similarly, in our previous study involving CRISPR/Cas9-mediated editing of the *SREBF1* locus (Aksoy et al. 2024), we demonstrated that deletion of the *SREBF1c* isoform affected the expression of genes acting upstream in the adipogenic cascade, including *CEBPB*. Notably, this disruption did not fully impair differentiation, which allowed for detailed functional analysis within the adipogenic network. These findings underscore the importance of gene selection in genome editing studies. Targeting genes that are upstream master regulators, may eliminate the possibility of studying adipogenesis altogether due to complete differentiation arrest. In contrast, focusing

on regulatory or downstream effectors—such as transcriptional modulators, co-factors, or isoforms—may enable more informative experiments that preserve cell viability and still providing mechanistic insight into adipose biology.

In conclusion, we demonstrated that CRISPR/Cas9-mediated deletion of the *CEBPB* promoter region resulted in a complete inhibition of adipogenic differentiation and a significant reduction in the proliferative potential of porcine mesenchymal stem cells. These findings indicate that *CEBPB* is essential not only for initiating adipogenesis but also for maintaining MSC proliferation. Therefore, in future experiments aimed at generating gene-edited pigs with modified adiposity, it is advisable to select target genes that do not completely impair both proliferation and differentiation processes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13353-025-01030-x.

Acknowledgements We would like to thank Dr. Tatiana Flisikowska (Technical University of Munich, Germany) for kindly providing the pX330 vector and Prof. Joanna Nowacka-Woszuk (Poznan University of Life Sciences, Poland) for the primers used for the analysis of cell proliferation marker genes.

Author contributions Conceptualization: M.O.A., J.R. M.S., and I.S.; funding acquisition: I.S.; investigation: M.O.A., J.R., M.S., and I.S.; methodology: M.O.A., J.R., M.S., and I.S; project administration: I.S.; resources: I.S.; supervision: I.S.; writing—original draft: M.O.A. and I.S.; writing—review and editing: M.O.A. and I.S. All authors have read and agreed to the published version of the manuscript.

Funding This study was financed by the Polish National Science Center, Grant No. 2018/29/B/NZ2/00956.

Declarations

Competing interests The authors declare no competing interests.

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Supplementary materials

The Role of the *CEBPB* Gene in Porcine Adipogenesis: A Study Using CRISPR/Cas9-Edited Mesenchymal Stem Cells

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Table S1 gRNAs/primers directed against the 5'flanking region of *CEBPB* gene.

	gRNA	Name	Sequence
CEBPB	gRNA5	CEBPb_g5_F	caccgCCGCGGCCGCGTTTAGGGT
		CEBPb_g5_R	aaacACCCTAAACGCGGCCGCGGc
	gRNA7	CEBPb_g7_F	caccgCGCGTTCATGCAACGCCTGG
		CEBPb_g7_R	aaacCCAGGCGTTGCATGAACGCGc

Table S2 Sequences of primers designed for genotyping single MSC colonies.

Primer name	Sequence	Annealing temperature
CEBPb_geno_F	GGGCTACCTCTCCAATAGCC	60°C
CEBPb_geno_R	AAGCAGTCCGCCTCGTAGTA	

16 **Table S3** List of primer pairs used in RT-qPCR.

Gene	Primer sequence	Product size (bp)	Annealing temperature (°C)	Genbank accession number
RPL27*	F: 5` GCAAAGCGGTCATCGTAAA R: 5` CTTGTGGGCATGAGGTGAT	190	60	NM_001097479.1
PPARG	F: 5` GCATCAGCTCTGTGGACCTG R: 5` GATCAGCTCTCGGGAATGGG	132	60	XM_005669783
CEBPA	F: 5` CGTGAGCGCAACAACATCG R: 5` CTCAGTTGTTCCACCCGCTT	131	60	NC_010448
FABP4	F: 5` TTCAAATTGGGCCAGGAAT R: 5` ATTCTGGTAGCCGTGACACC	191	60	NM_001002817
CEBPB	F: 5` TACTACGAGGCGGACTGCTT R: 5` TCCAGGTATGGGCTGAAGTC	152	60	NM_001199889.1
GATA2	F: 5` CTCCAGCTTCACCCCTAAG R: 5` CCCGTTCATCTTGTGGTACA G	157	60	XM_021068525.1
CCND1	F: 5` GCCGAGAAGTTGTGCATC TA	140	60	NC_010444

	R: 5` TTGGAGAGGAAGTGCTCG AT			
MCM2	F: 5` AGCATCGCTCCTTCCATCT A R: 5` CACAGGAGCACGTTGATG TC	128	60	NC_010455
PCNA	F: 5` GATTTAGATGTTGAGCAA CTTGG R: 5` GCACAGGAAATTACAACA GCA	131	60	NC_010459

* *reference gene*

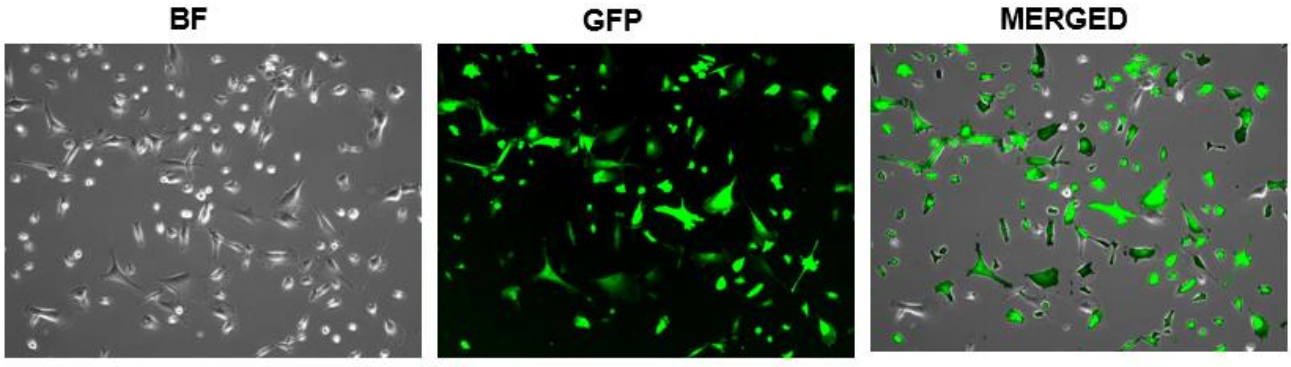

Fig. S1. Evaluation of transfection efficiency in MSCs using the pmaxGFP control plasmid (Lonza, Basel, Switzerland). Fluorescence microscopy images obtained 24 h after transfection show GFP expression in approximately 60% of cells, as determined by counting GFP-positive cells in five randomly selected microscopic fields. BF: bright field; GFP: green fluorescent protein channel; MERGE: fusion.

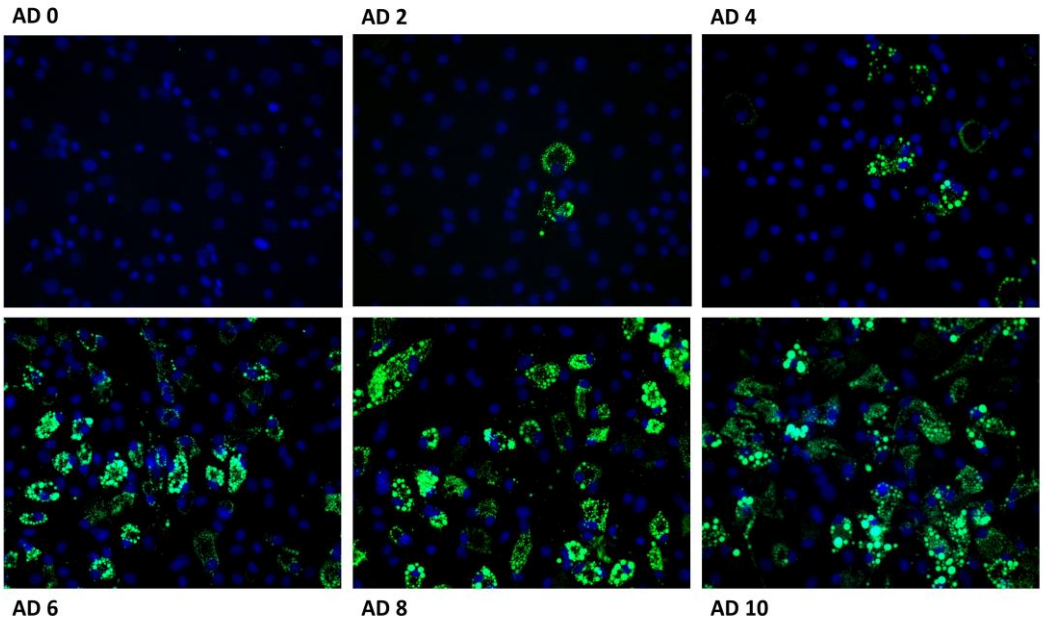

Fig. S2 BODIPY staining of wild-type mesenchymal stem cells (MSC_{WT}) showing progressive accumulation of lipid droplets during adipogenic differentiation (AD) on days 0, 2, 4, 6, 8, and 10 (AD0–AD10). In contrast, modified cells (MSC_{DEL}) failed to accumulate lipid droplets, indicating a lack of adipogenic potential following *CEBPB* promoter deletion.

genes

Article

Dynamic Histone Modification Patterns in Key Transcription Factor Genes During Porcine Adipogenesis

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Abstract

Background: Adipogenesis is governed by a complex interplay between transcriptional regulation and epigenetic remodeling. While many transcriptional pathways have been well characterized, less is known about how chromatin-level regulation shapes the timing of gene expression, particularly in large animal models such as pigs. In this study, we investigated histone modification patterns associated with four key adipogenic transcription factor genes—*PPARG*, *GATA2*, *CEBPA*, and *CEBPB*—in porcine mesenchymal stem cells (MSCs) undergoing adipogenic differentiation. Methods: Using RT-qPCR and ChIP-qPCR, we profiled gene transcription levels and epigenetic marks, including promoter- and exon-specific enrichment of the activating histone marks H3K9ac and H4K8ac, as well as the repressive mark H4K20me3, across six time points (day 0, 2, 4, 6, 8, and 10). Results: Although *PPARG* and *GATA2* are located in close proximity on porcine chromosome 13, they exhibited distinct histone modification profiles. *PPARG* showed progressive promoter acetylation (H4K8ac) accompanied by transcriptional activation, whereas *GATA2* displayed decreased exon acetylation (H3K9ac) associated with declining expression. In contrast, the H4K20me3 profile was similar for both genes, suggesting no direct association with their transcriptional activity. Interestingly, *CEBPA* (chromosome 6) and *CEBPB* (chromosome 17) exhibited temporally distinct histone modification patterns consistent with their roles in intermediate and early stages of adipogenic differentiation, respectively. Increased enrichment of the H3K9ac mark preceded the rise in transcript levels of the analyzed genes. Promoter regions showed higher enrichment of H4K8ac compared with exonic regions. A higher level of H4K20me3 was also observed for *CEBPA* and *CEBPB* than for *PPARG* and *GATA2*, which appeared to be more related to chromosomal localization than to direct transcriptional regulation. Conclusions: Together, these results reveal complex interactions between transcriptional dynamics and selected histone modifications that depend on both the gene analyzed and the stage of adipocyte differentiation. This study provides new insights into the epigenetic regulation of porcine adipogenesis and highlights chromatin context as an additional layer influencing transcriptional control.

Keywords: adipocytes; ChIP-qPCR; histone marks; mesenchymal stem cells; pig

Academic Editor: Katarzyna Piórkowska

Received: 25 March 2026
Revised: 19 April 2026
Accepted: 24 April 2026
Published: 28 April 2026

1. Introduction

Adipogenesis, the process by which precursor cells differentiate into mature adipocytes, plays a crucial role in several biological processes, including energy homeostasis and lipid metabolism. This process is tightly regulated and involves a complex network of molecular pathways controlled by multiple regulatory elements [1]. Within this intricate regulation,

numerous transcription factors and epigenetic regulators act in a coordinated manner, contributing to either the activation or repression of adipogenic gene expression. Among the key transcription factors that govern adipocyte differentiation, peroxisome proliferator-activated receptor gamma (PPARG), CCAAT/enhancer-binding proteins (CEBPA and CEBPB), and GATA Binding Protein 2 (GATA2) are well known for their critical roles in the initiation and progression of adipogenesis. While PPARG and members of the CEBP family promote the expression of pro-adipogenic genes, GATA2 is recognized as a negative regulator that inhibits adipogenic gene expression and thereby acts as a suppressor of adipocyte differentiation [2–4].

Recent studies have demonstrated that transcriptional regulation during adipogenesis is not solely governed by transcription factors but is also strongly influenced by epigenetic mechanisms, including chromatin structure and histone modifications [5–8]. Epigenetic marks such as histone acetylation and methylation dynamically change throughout the differentiation process, modulating the accessibility of chromatin and the transcriptional activity of key adipogenic regulators [9–11]. Histone acetylation marks associated with euchromatin, such as H3K9ac and H4K8ac, are generally linked to transcriptional activation and an open chromatin conformation; however, their presence does not necessarily indicate active transcription in all contexts, as they may also reflect a more permissive chromatin state or be involved in processes other than direct gene activation. In contrast, trimethylation marks like H4K20me3, H3K27me3 and H3K9me3 are strongly associated with heterochromatin, and correlated with transcriptional repression, gene silencing and chromatin compaction [8,12,13]. During adipocyte differentiation, chromatin undergoes substantial structural remodeling driven by dynamic changes in histone post-translational modifications. Repressive H4K20me3 is progressively enriched at genomic regions undergoing transcriptional silencing, supporting heterochromatin formation and reduced accessibility during lineage commitment [14]. In contrast, activating acetylation marks such as H3K9ac and H4K8ac promotes transcription initiation by loosening nucleosome packing and facilitating transcription factor binding to adipogenic regulatory elements [8,15]. These activating marks are particularly associated with enhanced expression of master regulators, including *PPARG* and *CEBPA*, as preadipocytes transition to a mature adipocyte state [8]. Importantly, studies in porcine mesenchymal stem cell-derived adipocytes have demonstrated that the balance between H4K20me3 accumulation and the elevation of H3K9ac/H4K8ac closely reflects stage-specific chromatin accessibility and activation of adipogenesis [10]. Together, these observations highlight that assessing both permissive and repressive histone signatures is essential for understanding transcriptional control mechanisms that drive adipogenesis. Understanding the distribution and correlation of these histone modifications between promoter and exon regions of adipogenic genes provides valuable insights into the epigenetic mechanisms that regulate this differentiation process.

Previous research has reported that histone modifications play a role in the activation of *PPARG* and *CEBPB*, whereas regulatory factors such as *GATA2* exhibit an opposing epigenetic profile, acting as suppressors of adipogenic differentiation [1,16,17]. Most of these findings originate from murine systems, particularly studies performed in the 3T3-L1 mouse preadipocyte cell line, where acetylation marks at *PPARG* and *CEBPB* promoters have been associated with enhanced adipogenic transcription, while repressive methylation marks at *GATA2* loci have been linked to suppression of adipocyte maturation [17]. Similar regulatory patterns have also been observed in human primary preadipocytes and murine models, supporting the idea that chromatin remodeling is essential for adipogenesis [1]. Broader epigenetic analyses in mammals, including human and murine studies, also indicate that histone-based regulation is a conserved feature of adipocyte differentiation [16]. Although the regulation of adipogenesis shares many conserved mech-

anisms across species, chromatin dynamics and epigenetic architecture can differ, especially in livestock animals. In pigs, adipose tissue biology is not only important for metabolic research but also directly linked to fat deposition and meat quality traits [18,19]. Moreover, pigs display strong metabolic and endocrine similarities to humans, making them highly informative models for investigating obesity, insulin resistance, and other metabolic disorders [20]. Their adipocyte development and lipid metabolism closely resemble human physiology, including preadipocyte maturation [21]. In our previous study, we examined the abundance and nuclear distribution of selected heterochromatin (H3K9me3, H3K27me3, and H4K20me3) and euchromatin (H4K8ac, H3K4me3, and H3K9ac) histone marks during in vitro adipogenesis [10]. However, that study focused primarily on global changes in histone mark distribution and did not address gene-specific regulatory regions. Therefore, examining promoter- and exon-specific histone modifications in porcine mesenchymal stem cells provides novel biological insight and helps bridge the existing knowledge gap between human, mouse, and livestock adipogenesis research.

In the present study, we aimed to investigate the relationships between histone modifications (H4K20me3, H4K8ac, and H3K9ac) within the promoter and exon regions of several key adipogenic genes (*PPARG*, *GATA2*, *CEBPA*, and *CEBPB*) during adipocyte differentiation from porcine mesenchymal stem cells. *PPARG* and *GATA2*, which are located on the same chromosome and exhibit antagonistic regulatory activity, were analyzed alongside *GP9*, a gene located on the same chromosome but not associated with adipogenesis, which served as a control. To address this objective, we performed ChIP-qPCR analyses targeting three major histone modifications. These epigenetic measurements were complemented by transcriptional profiling using real-time PCR, followed by correlation analysis to determine how chromatin signatures align with gene activation or repression throughout adipocyte maturation. Samples were collected at six defined time points (day 0, 2, 4, 6, 8, and 10). Understanding these relationships improves our knowledge of adipogenesis and provides insight into how chromatin changes influence transcriptional regulation during adipocyte differentiation.

2. Materials and Methods

2.1. Cell Culture and Adipogenesis Induction

Adipose-derived mesenchymal stem cells (AD-MSC) were isolated from adipose tissue collected post mortem from Large White pigs at a commercial slaughterhouse. As no experimental procedures were conducted on live animals, ethical approval was not required in accordance with applicable regulations. AD-MSC were cultured in Advanced DMEM medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% FBS (Sigma-Aldrich, Darmstadt, Germany), 5 ng/mL FGF-2 (PromoKine, Heidelberg, Germany), 2 mM L-Glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1 mM 2-mercaptoethanol (Sigma-Aldrich, Darmstadt, Germany), 1× antibiotic-antimycotic solution (Sigma-Aldrich, Darmstadt, Germany), and 1× MEM NEAA (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). These cells were maintained at 37 °C in a 5% CO_2 atmosphere. Once the cells reached confluency, adipogenesis was initiated using a differentiation medium. This differentiation medium consisted of Advanced DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 10% FBS (Sigma-Aldrich, Darmstadt, Germany), 1× antibiotic-antimycotic solution (Sigma-Aldrich, Darmstadt, Germany), 5 ng/mL FGF-2 (PromoKine, Heidelberg, Germany), 1× Linoleic Acid Albumin (Sigma-Aldrich, Darmstadt, Germany), 1× ITS Supplement (Sigma-Aldrich, Darmstadt, Germany), 1 µM Dexamethasone (Sigma-Aldrich, Darmstadt, Germany), 100 µM Indomethacin (Sigma-Aldrich, Darmstadt, Germany), and 50 µM IBMX (Sigma-Aldrich, Darmstadt, Germany). The differentiation process was conducted for ten days, with the medium being changed

every 24 h. The progression of adipogenesis was monitored by observing lipid droplet formation under phase-contrast microscopy (TS100 Eclipse, Nikon, Melville, NY, USA) and by BODIPY (Life Technologies, Grand Island, NY, USA) staining, following the protocol described previously by Aksoy et al. [22].

2.2. RNA Isolation, cDNA Synthesis, and Real-Time PCR

Total RNA was isolated from AD-MSC cell cultures and differentiating cells utilizing the TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany). Following thorough quantitative and qualitative assessments with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), one microgram of RNA was then converted into complementary DNA (cDNA) using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Germany). For real-time PCR, specific primers were designed for each target transcript. These primers were designed to bind to distinct exons, referencing the Sscrofa 11.1 sequence (details in Table S1). Primer efficiency was determined based on standard curves, and specificity was confirmed by melt curve analysis. All PCR reactions were conducted in triplicate on a LightCycler 480 II instrument (Roche, Basel, Germany) employing the LightCycler 480 SYBR Green I Master Kit (Roche, Basel, Germany). The relative messenger RNA (mRNA) levels of the genes under investigation were determined via the second derivative maximum method. Finally, the obtained results were normalized against the geometric mean expression of the reference genes, *RPL27* and *PPIA*.

2.3. ChIP-qPCR Analysis

Chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) was performed to characterize histone modification enrichment at promoter and exon regions of adipogenic genes throughout differentiation. The procedure was carried out using the iDeal ChIP-seq Kit for Histones (Diagenode, Liège, Belgium; Kit Cat# C01010051) according to the manufacturer's instructions, with minor adjustments optimized for porcine mesenchymal stem cells (AD-MSC). Cells were harvested at six differentiation time points (days 0, 2, 4, 6, 8, and 10), and culture medium was replaced daily to maintain consistent adipogenic induction. Cells were cross-linked with 1% formaldehyde for 8 min at room temperature, followed by quenching with 0.125 M glycine for 5 min. After two washes with ice-cold PBS, cells were scraped and lysed using SDS Lysis Buffer. Chromatin was sheared to ~100–500 bp fragments by sonication using a Bioruptor (Diagenode, Liège, Belgium). Following centrifugation at 14,000× *g* for 10 min at 4 °C, the soluble chromatin fraction was collected for immunoprecipitation. Immunoprecipitation was performed overnight at 4 °C using antibodies against H4K20me3 (Rabbit polyclonal, Abcam, Cambridge, UK; Cat# ab9053), H4K8ac (Rabbit polyclonal, Abcam, Cambridge, UK; Cat# ab15823), and H3K9ac (Rabbit polyclonal, Abcam, Cambridge, UK; Cat# ab10812) (Table S2). Rabbit IgG provided within the kit was used as a negative control. Immunocomplexes were retrieved using Protein A magnetic beads, washed sequentially to remove nonspecific binding, and eluted with the Elution Buffer supplied in the kit. Cross-links were reversed at 65 °C overnight and DNA was purified using silica-based spin columns. Quantitative PCR was performed using the LightCycler 480 II instrument (Roche, Basel, Germany) and the LightCycler 480 SYBR Green I Master Kit (Roche, Basel, Germany). Primers were designed to specifically amplify promoter and exon regions of *PPARG*, *GATA2*, *GP9*, *CEBPA*, and *CEBPB* (Table S1). Each 20 µL reaction contained 1 µL of immunoprecipitated DNA and was run in technical replicates. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Melting-curve analysis confirmed amplification specificity. Relative enrichment was calculated using

the ΔCt method and normalized to input DNA, with data expressed as % input. Results represent mean ± standard deviation (SD) from three independent biological replicates.

2.4. Statistical Analysis

All statistical analyses were performed in R version 4.4.2 (R Foundation for Statistical Computing, Vienna, Austria). Associations between ChIP–qPCR enrichment (% recovery) and relative gene expression were evaluated separately for each gene, genomic region, and histone mark using Spearman's rank correlation coefficient (ρ), as a non-parametric approach suitable for small sample sizes and potential non-linear relationships. For each analysis, the number of complete paired observations, correlation coefficient, and corresponding *p*-value were calculated. *p*-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) procedure across all correlation tests. Correlation analyses were conducted only when at least three complete paired observations were available and when variability was present in both variables

3. Results

3.1. Verification of Adipogenic Differentiation by BODIPY Staining

Adipogenic differentiation of porcine mesenchymal stem cells was verified by BODIPY staining, which enabled visualization of intracellular lipid droplets during the differentiation process (Figure 1). Minimal lipid accumulation was observed at day 0, whereas small lipid droplets became detectable at day 2 and day 4. A marked increase in lipid droplet formation was observed at later stages of differentiation, particularly from day 6 onwards. By day 8 and day 10, extensive lipid accumulation was evident in the majority of cells (Figure S1). These observations confirmed successful adipogenic differentiation under the applied culture conditions.

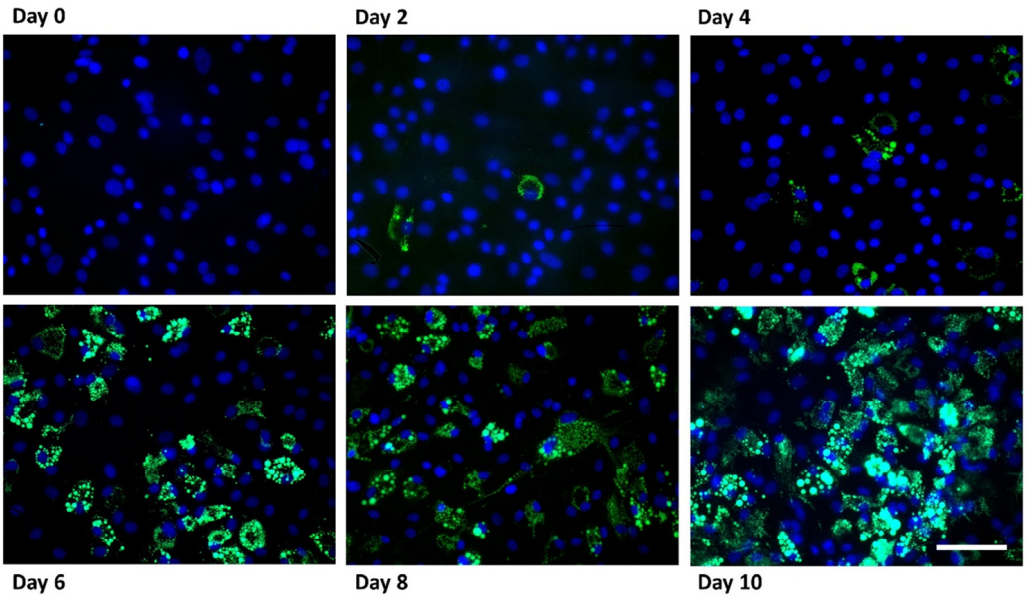

Figure 1. BODIPY staining during adipogenic differentiation of porcine mesenchymal stem cells. Representative fluorescence images showing intracellular lipid droplet accumulation at different stages of adipogenic differentiation (day 0, 2, 4, 6, 8 and 10). Lipid droplets are stained with BODIPY (green) and nuclei are counterstained with DAPI (blue). Scale bar: 50 μm.

3.2. Genomic Location of the Studied Genes

In *Sus scrofa*, the genomic organization of the analyzed genes demonstrates substantial diversity in both chromosomal location and gene size. The *PPARG* gene is positioned on chromosome 13 at chr13: 68,302,322–68,433,944, spanning 131,622 bp. On the same

chromosome, the negative regulator *GATA2* is located at chr13: 71,984,067–71,997,619 with a total length of 13,552 bp, whereas the non-adipogenic control gene *GP9* resides nearby at chr13: 71,560,012–71,562,774 and spans 2762 bp (Sscrofa11.1 assembly). *CEBPA* is positioned on chromosome 6 at chr6: 43,083,207–43,085,836, spanning 2630 bp, while *CEBPB* resides on chromosome 17 at chr17: 51,722,426–51,724,305, spanning 1880 bp (Sscrofa11.1 assembly) (Figure 2). These genomic coordinates were retrieved from the Ensembl database (Sscrofa11.1 assembly). The heterogeneity in chromosomal positioning, gene size, and functional roles among these loci provides a strong rationale for investigating their region-specific chromatin states and histone modification dynamics during adipogenesis.

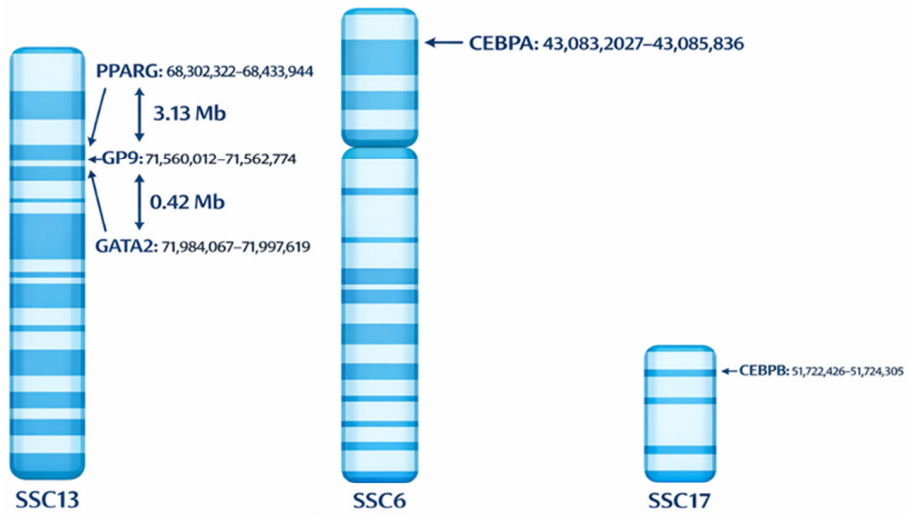

Figure 2. Schematic representation of the genomic locations of and *PPARG*, *GP9* and *GATA2* on chromosome 13 (SSC13), *CEBPA* on chromosome 6 (SSC6) and *CEBPB* on chromosome 17 (SSC17) (Sscrofa11.1). The diagram illustrates the chromosomal spacing between loci to highlight their genomic positions.

3.3. Dynamics of Gene Expression and Histone Modifications of PPARG and GATA2 During Adipogenesis

PPARG expression demonstrated a clear temporal increase throughout adipogenic differentiation (Figure 3A). The transcription levels were low at day 0, followed by a gradual elevation at days 2 and 4. A more pronounced upregulation was detected at day 6, with maximal transcript abundance observed at day 8 and day 10.

Analysis of histone modifications revealed dynamic changes at both promoter and exon regions of the *PPARG* locus. H4K20me3 enrichment remained low and relatively stable from day 0 to day 6 in both regions (Figure 3B). Beginning at day 8, enrichment levels increased, with the higher value observed for promoter site. H4K8ac enrichment was very low during early and intermediate stages (day 0–day 6), and the profiles were comparable in both promoter and exon regions (Figure 3C). A marked increase was observed at day 8, which persisted or further increased at day 10 in the promoter region. H3K9ac displayed a high level compared to the 2 previous modifications (Figure 3D). An early enrichment peak was observed at day 2 in both promoter and exon regions. Following this peak, enrichment decreased. Overall, the *PPARG* locus exhibited stage-dependent alterations in activating (H4K8ac, H3K9ac) and repressive (H4K20me3) histone marks across promoter and exon regions, with the most pronounced changes observed for histone acetylation.

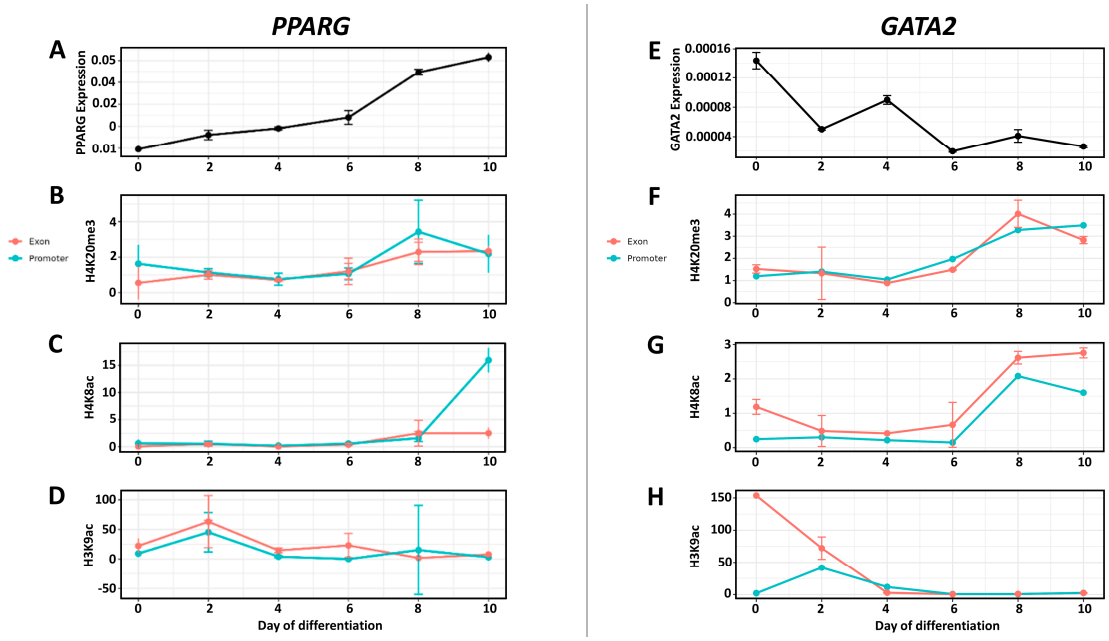

Figure 3. Gene expression and histone modifications of *PPARG* and *GATA2* during adipogenic differentiation. Relative mRNA expression and histone mark enrichment across differentiation stages (day 0, 2, 4, 6, 8, and 10) in porcine mesenchymal stem cells. The left panels correspond to *PPARG*, while the right panels correspond to *GATA2*. (**A**,**E**) Relative gene expression determined by RT-qPCR. (**B**,**F**) H4K20me3 enrichment at promoter and exon regions. (**C**,**G**) H4K8ac enrichment at promoter and exon regions. (**D**,**H**) H3K9ac enrichment at promoter and exon regions. Histone mark enrichment was measured by ChIP-qPCR and expressed as a percentage of input DNA. Data are presented as mean ± SD of biological replicates.

GATA2 expression was highest at day 0 and decreased markedly following induction of adipogenic differentiation (Figure 3E). A pronounced reduction was observed at day 2. Although a transient increase was detected at day 4, expression levels remained lower than baseline and continued at relatively reduced levels to the end of differentiation. Analysis of histone modification enrichment revealed stage-dependent alterations at both promoter and exon regions of the *GATA2* locus. H4K20me3 enrichment remained low and relatively stable from day 0 to day 6 in both regions (Figure 3F). An increase was observed at day 8 and day 10, with higher enrichment levels detected at the promoter compared to the exon at late stages. H4K8ac levels were generally low during early differentiation stages (Figure 3G). A moderate increase was observed at day 8 and day 10, particularly in the exon region. H3K9ac displayed elevated enrichment at day 0 in the exon region, followed by a substantial decrease beginning at day 2 (Figure 3H). H3K9ac levels in the promoter remained comparatively low throughout differentiation, with a peak at day 2.

To determine whether chromatin remodeling patterns observed at adipogenic transcription factor loci were gene-specific, histone modification enrichment was also examined at the *GP9* locus, a non-adipogenic gene located on the same chromosome as *PPARG* and *GATA2*. H4K20me3 enrichment displayed dynamic variation across differentiation stages (Figure S2A), with elevated levels at early stages and a pronounced peak at day 4, particularly in the exon region. Enrichment decreased substantially at later stages (day 6–day 8) before partially increasing again at day 10. H4K8ac levels showed moderate enrichment across early and intermediate stages in both promoter and exon regions (Figure S2B), followed by a decline at day 10. In contrast, H3K9ac enrichment was highest at day 0, particularly in the exon region, and decreased sharply thereafter, while promoter-associated enrichment remained low throughout differentiation (Figure S2C). Overall, the *GP9* locus

exhibited temporal variation in histone modification enrichment during adipogenic differentiation. These results indicate that although the three genes (*PPARG*, *GP9* and *GATA2*) are located close to each other, each exhibits a distinct histone modification profile.

Taken together, in *PPARG* and *GATA2* genes, variable histone acetylation patterns was observed in the promoter and exon regions, suggesting that this modification may act as a regulatory component of these genes. In contrast, H4K20me3 did not show a clear correlation with gene expression, and changes in this repressive modification appear to be less directly associated with transcription levels.

3.4. Dynamics of Gene Expression and Histone Modifications of CEBPA and CEBPB During Adipogenesis

CEBPA expression displayed a distinct temporal profile during adipogenic differentiation (Figure 4A). Expression levels gradually increased from day 0 to day 4, followed by a more pronounced elevation at day 6, where peak transcript abundance was observed. After day 6, expression levels declined at day 8 and day 10, although remaining above baseline levels detected at day 0.

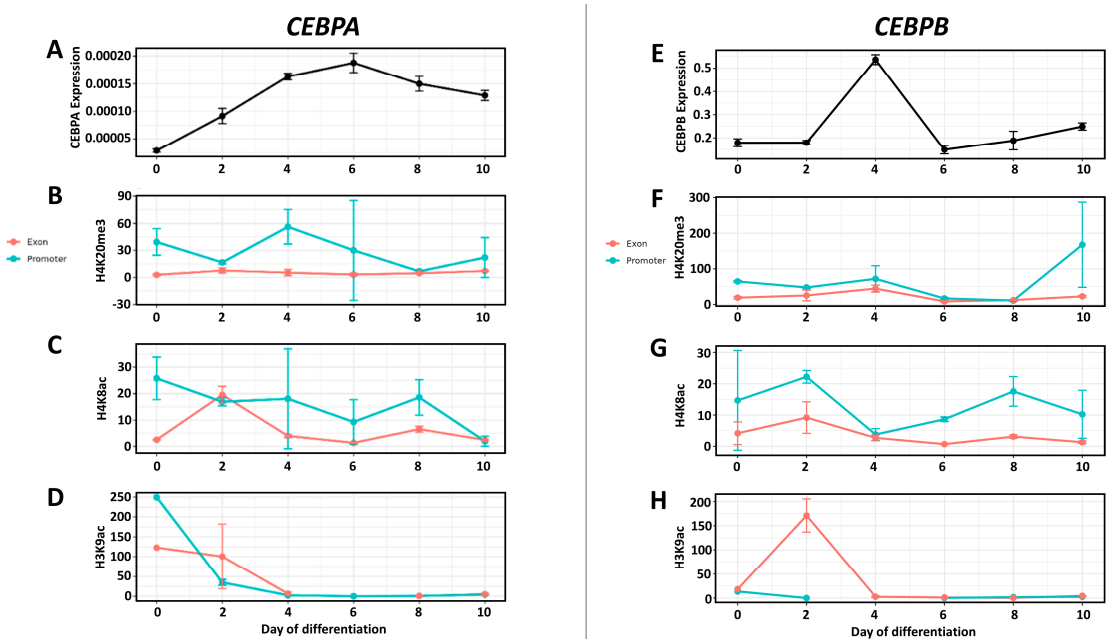

Figure 4. Gene expression and histone modification dynamics of *CEBPA* and *CEBPB* during adipogenic differentiation. Relative mRNA expression and histone mark enrichment across differentiation stages (day 0, 2, 4, 6, 8, and 10) in porcine mesenchymal stem cells. The left panels correspond to *CEBPA*, while the right panels correspond to *CEBPB*. (**A**,**E**) Relative gene expression determined by RT-qPCR. (**B**,**F**) H4K20me3 enrichment at promoter and exon regions. (**C**,**G**) H4K8ac enrichment at promoter and exon regions. (**D**,**H**) H3K9ac enrichment at promoter and exon regions. Histone mark enrichment was measured by ChIP-qPCR and expressed as a percentage of input DNA. Data are presented as mean ± SD of biological replicates.

Histone modification analysis revealed dynamic and region-specific changes across the *CEBPA* locus. H4K20me3 enrichment showed a sharp increase at the promoter region on day 4 (Figure 4B). Outside of this time point, promoter enrichment levels remained low throughout the differentiation stages. In contrast, exon-associated H4K20me3 levels exhibited minimal variation over time. H4K8ac enrichment at the exon was elevated on day 2, followed by a gradual decline (Figure 4C). In comparison, H4K8ac levels at the promoter were generally lower and displayed less pronounced temporal variation than those observed in the exon region. H3K9ac enrichment was highest at day 0, particularly

in the promoter region, and markedly decreased by day 2 (Figure 4D). From day 4 onward, low levels were maintained in both the promoter and exon regions throughout the differentiation process.

CEBPB expression exhibited a transient and dynamic pattern during adipogenic differentiation (Figure 4E). Expression levels were low at day 0 and day 2, followed by a marked increase at day 4. After reaching this peak, transcript levels declined at day 6 and remained at lower levels at day 8 and day 10. Histone modification profiling demonstrated stage-specific and region-dependent changes at the *CEBPB* locus. H4K20me3 enrichment remained relatively stable and comparable for both studied genomic regions throughout the differentiation timeline, with only a peak at day 10 for promoter (Figure 4F). H4K8ac enrichment showed comparable profile for both exon and promoter, with small fluctuations more visible for promoter (Figure 4G). The level of this modification was low compared to the other two. H3K9ac demonstrated a distinct enrichment pattern in the exon region, with a marked peak at day 2 (Figure 4H). Following this early increase, enrichment levels declined and remained low across subsequent differentiation stages. Promoter H3K9ac levels remained minimal throughout the time course.

A summary of these results suggests that increases in H4K8ac and/or H3K9ac occur before or coincide with increases in gene expression, indicating that histone acetylation may prime chromatin for transcriptional activation. At many time points, the promoter region exhibits higher levels of acetylation than the exon region, suggesting that epigenetic regulation occurs primarily at the promoter level. In contrast, H4K20me3 does not show a clear correlation with gene expression levels, and its changes appear to be less closely associated with transcriptional activity than those of histone acetylation.

3.5. Correlation Between Histone Mark Enrichment and Gene Expression

Spearman's rank correlation analysis was performed to evaluate the association between histone mark enrichment and gene expression levels across differentiation stages. To account for multiple testing, *p*-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) procedure. Overall, although several strong monotonic trends were observed, none of the correlations reached statistical significance after multiple testing correction (all FDR $\geq$ 0.200). Therefore, these results should be interpreted with caution and are considered exploratory. A comprehensive overview of all correlation coefficients and corresponding FDR-adjusted *p*-values is provided in the Supplementary Materials (Figure S3). In general, stronger correlations were observed in exon regions than in promoter regions, which may suggest a potential role of the analyzed histone modifications in regulating chromatin structure within the gene body.

4. Discussion

Adipose tissue differentiation is a complex biological process characterized by dynamic chromatin remodeling and tightly regulated transcriptional events. In this study, we investigated temporal patterns of histone modifications in the promoter and exon regions of four key adipogenic transcription factors in porcine mesenchymal stem cells and evaluated their association with gene expression during differentiation. Histone modifications constitute a major epigenetic mechanism regulating chromatin accessibility and transcriptional competence during cellular differentiation. In adipogenesis, activating histone marks, such as acetylation, are generally associated with open chromatin and transcriptional activation, whereas repressive methylation marks are linked to chromatin compaction and transcriptional silencing. Therefore, dynamic changes in these modifications are expected to contribute to the coordinated activation of pro-adipogenic genes and repression of anti-adipogenic regulators [1,13,16].

Our results demonstrated that histone modifications are dynamically regulated over time during adipogenesis, exhibiting gene-specific temporal patterns. The observed differences in the timing and rate of these changes suggest that epigenetic regulation is tightly coordinated with the progression of the differentiation program and may contribute to stage-specific gene expression. The progressive upregulation of *PPARG* observed in our model is consistent with its established role as a central regulator of terminal adipocyte differentiation [1,23]. Previous genome-wide studies in murine and human systems have demonstrated increased acetylation at the *PPARG* locus during adipogenesis, particularly enrichment of H3K9ac and H3K27ac at regulatory regions [11,24]. In agreement with these findings, we observed late-stage elevation of H4K8ac at both promoter and exon regions of *PPARG*, coinciding temporally with maximal transcript levels. Our study highlights the importance of H4K8ac enrichment in porcine adipogenesis through gene- and region-specific analysis. *GATA2*, known to function as an anti-adipogenic factor that must be downregulated for adipocyte commitment [17,25], displayed declining expression following induction of differentiation. This transcriptional repression was accompanied by reduced H3K9ac enrichment at early stages and increased H4K20me3 at later time points, suggesting a shift toward a more repressive chromatin state. Similar epigenetic transitions have been reported in murine adipogenesis models, where loss of activating acetylation marks at anti-adipogenic loci facilitates lineage progression [1,16]. An additional point of interest in the present study is that *PPARG* and *GATA2* are located on the same porcine chromosome, yet they displayed clearly distinct temporal patterns of histone modification during adipogenesis. This observation suggests that chromosomal proximity alone does not impose uniform epigenetic regulation, and that local chromatin context may be more relevant than linear genomic distance. This may reflect locus-specific regulatory features, such as differences in chromatin accessibility, transcription factor binding, or local regulatory element activity. Consequently, genes located within the same chromosomal region may still undergo distinct epigenetic regulation depending on their local regulatory environment. However, no direct chromatin conformation data were generated in this study, and therefore this interpretation requires further validation. This issue is particularly important in pigs, as although many adipogenesis-related genes are evolutionarily conserved, their chromosomal localization differs between pig and human, which may influence higher-order chromatin organization and regulatory interactions [26]. *CEBPA* exhibited peak expression at intermediate stages, in agreement with its established role in consolidating adipocyte identity [27]. The region-specific nature of this association underscores the importance of distinguishing promoter and exon chromatin states, as gene body and promoter acetylation may reflect distinct regulatory processes [28]. The transient induction of *CEBPB* at early stages of differentiation aligns with its established function as an early adipogenic initiator [29]. Correspondingly, we observed early promoter-associated acetylation, particularly H4K8ac enrichment at day 2, preceding peak transcript levels. This pattern is consistent with reports that *CEBPB* activation is accompanied by rapid chromatin remodeling during mitotic clonal expansion in 3T3-L1 cells [30].

Our analyses indicate that histone modification dynamics are not uniformly distributed across gene regions at the examined loci. This interpretation is supported by the observation that promoter- and exon-associated enrichment patterns frequently differed in both magnitude and temporal trajectory. Such region-specific behavior is consistent with genome-wide chromatin studies demonstrating functional compartmentalization of histone modifications between regulatory and coding regions [12,28]. The distinction between promoter- and gene body-associated histone modifications is particularly relevant in the context of transcriptional regulation. Promoter-associated acetylation is often linked to transcriptional initiation and recruitment of regulatory complexes, whereas histone

marks within gene bodies may reflect transcriptional elongation, chromatin accessibility, or broader structural organization of active loci. In this context, the differences observed between promoter and exon regions in our study support the view that region-specific chromatin profiling provides more informative insight than gene-level analysis alone [1,12,28]. This spatial heterogeneity suggests that promoter-centered chromatin remodeling may play a primary role in transcriptional initiation, whereas exon-associated marks may reflect transcriptional elongation or broader chromatin organization. The temporal differences observed between promoter- and exon-associated histone modifications suggest that distinct chromatin regions may be differentially regulated at specific stages of adipogenesis, contributing to the precise coordination of gene expression programs during differentiation.

Beyond linear gene organization, chromatin is arranged in three-dimensional structures that include loops bringing distal regulatory elements into spatial proximity with promoters. These chromatin loops may span from kilobases to megabases and are dynamically reorganized during differentiation. Histone modifications are thought to contribute to loop formation and stabilization by altering chromatin accessibility and nucleosome organization [31,32]. Therefore, if genes located on the same chromosome undergo different spatial repositioning during adipogenesis, they may also acquire distinct local histone modification patterns. This may help explain why *PPARG* and *GATA2*, despite their chromosomal proximity, exhibited different epigenetic profiles in the present study [26,33,34]. The inclusion of *GP9* as a non-adipogenic locus located on the same chromosome as *PPARG* and *GATA2* enabled a partial assessment of genomic context effects. Although temporal variation in histone mark enrichment was observed at the *GP9* locus, the absence of transcriptional profiling prevented evaluation of expression–chromatin coupling. Nevertheless, the presence of chromatin dynamics at this locus suggests that differentiation-associated epigenetic remodeling may extend beyond strictly adipogenic genes.

Our findings should also be interpreted in the context of previous porcine studies on adipogenic epigenetic regulation. Stachecka et al. [10] demonstrated dynamic alterations in active and repressive histone marks at the protein level during adipogenic differentiation of porcine mesenchymal stem cells using Western blot analysis. Although our study assessed locus-specific enrichment by ChIP-qPCR rather than global protein abundance, both datasets support the view that adipogenesis in porcine cells is accompanied by coordinated remodeling of activating and repressive histone signatures. In addition, Kociucka et al. [35] reported correlations between histone modifications and expression of key adipocyte-related genes in porcine adipose tissue, further supporting the biological relevance of histone-mediated regulation in pigs [10,35]. Therefore, while temporal concordance between chromatin modification and transcriptional activity was observed, our findings should be interpreted as descriptive of dynamic association rather than conclusive evidence of direct regulatory coupling. It is also worth noting that epigenetic regulation of adipogenesis may be influenced by environmental and nutritional factors. In particular, nutritional compounds capable of modulating histone deacetylase activity may affect adipocyte differentiation and lipid accumulation. In porcine adipose tissue, short-chain fatty acids have been shown to enhance adipocyte differentiation, highlighting the possibility that metabolic inputs may interact with chromatin-level regulation during adipogenesis [36].

Future studies employing genome-wide epigenomic profiling approaches and increased temporal resolution will be necessary to more precisely define chromatin–transcription relationships during porcine adipogenesis. In particular, integration of genome-wide chromatin profiling techniques such as ChIP-seq or ATAC-seq would enable the identification of additional regulatory elements and facilitate evaluation of whether comparable epigenetic patterns are conserved across species [37,38]. Moreover, emerging methods for analysis

of higher-order chromatin architecture and chromatin conformation [39,40] may provide further insight into how three-dimensional genome organization contributes to adipogenic gene regulation.

5. Conclusions

This study provides insight into histone modifications at key adipogenic transcription factor genes during the differentiation of porcine mesenchymal stem cells. By integrating gene expression profiling with promoter- and exon-specific analyses of histone marks, we identified dynamic, stage-dependent changes in activating (H3K9ac, H4K8ac) and repressive (H4K20me3) histone modifications at adipogenic loci. The temporal patterns of histone modifications only partially paralleled transcriptional dynamics, indicating complex chromatin remodeling throughout differentiation. Notably, histone modification dynamics were frequently region-specific, with promoter and exon regions displaying distinct enrichment trajectories. Overall, our findings support a model in which adipogenic differentiation is accompanied by coordinated, yet gene- and region-specific, chromatin remodeling.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes17050521/s1. Table S1. Primer sequences, amplicon lengths, and annealing temperatures used for qPCR and ChIP-qPCR amplification of promoter and exon regions of target genes. Table S2. Antibodies used for ChIP-qPCR, including target specificity, host species, provider information, catalog numbers, and amounts applied per immunoprecipitation. Figure S1. Lipid accumulation during adipogenic differentiation assessed by BODIPY fluorescence intensity. A gradual increase in lipid accumulation was observed over time, with a marked elevation from day 6 onwards and a peak at day 10. Data represent mean fluorescence intensity values corresponding to each time point. Figure S2. Histone modification profiles at the GP9 locus during adipogenic differentiation. Enrichment of H4K20me3 (A), H4K8ac (B), and H3K9ac (C) at promoter and exon regions of the GP9 locus across six differentiation stages (from day 0 to day 10) in porcine mesenchymal stem cells. Histone mark enrichment was determined by ChIP-qPCR and expressed as percentage of input DNA. Figure S3. Spearman correlation analysis between histone mark enrichment and gene expression. Heatmap representation of Spearman correlation coefficients (ρ) between histone mark enrichment (H4K20me3, H4K8ac, and H3K9ac) at promoter and exon regions and corresponding gene expression levels across differentiation stages. Correlation coefficients and FDR-adjusted *p*-values (Benjamini–Hochberg correction) are indicated within each cell. No correlations reached statistical significance after multiple testing correction.

Author Contributions: Conceptualization: M.O.A. and I.S.; methodology: M.O.A., J.W., M.S. and I.S.; formal analysis: M.O.A., J.W. and I.S.; investigation: M.O.A., J.W. and I.S.; writing—original draft preparation: M.O.A.; writing—review and editing: M.O.A. and I.S.; visualization: M.O.A. and J.W.; supervision: I.S.; project administration: I.S.; funding acquisition: I.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financed by the Polish National Science Center, Grant No. 2018/29/B/NZ2/00956 and by the statutory fund of the Department of Genetics and Animal Breeding, Faculty of Veterinary Medicine and Animal Science, Poznan University of Life Sciences, Poland (No. 506.534.09.00).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of the study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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Supplementary material

Table S1. Primer sequences, amplicon lengths, and annealing temperatures used for qPCR and ChIP-qPCR amplification of promoter and exon regions of target genes.

Primer Names	Sequences	Amplicon length	Annealing Temprature
*CEBPA*_Exon	F: 5` CGTGAGCGCAACAACATCG 3` R: 5` CTCAGTTGTTCCACCCGCTT 3`	131 bp	60°C
*CEBPA*_Promoter	F: 5' TGGGGAAGCCAGAGGAGAG 3` R: 5' GTTAGAGGGCGGTGAGTGG 3`	181 bp	60°C
*CEBPB*_Exon	F: 5` TACTACGAGGCGGACTGCTT 3` R: 5` TCCAGGTATGGGCTGAAGTC 3`	152 bp	60°C
*CEBPB*_Promoter	F: 5' AAAGCCCCCAACCACAAGTC 3` R: 5' TCAAGATCAGCGGCTCTCAG 3`	233 bp	60°C
*PPARG*_Exon	F: 5' GGATCAGCTCTGTGGACCTG 3` R: 5' GATCAGCTCTCGGGAATGGG 3`	132 bp	60°C
*PPARG*_Promoter	F: 5' CAGGATGCGCTGGTGTTTG 3` R: 5' CTTCTGACCGAGCCTGACTC 3`	104 bp	60°C
GATA2 Exon	F: 5' CACACTTGTTGCACAGCCC 3` R: 5' CTTGGAGAAGGGGTTGACGG 3`	110 bp	60°C
GATA2 Promoter	F: 5' TCTGTGAACAGGCAGCAGTC 3` R: 5' ACCTTGGGCCCAAACAGAAA 3`	137 bp	60°C
GP9 Exon	F: 5' GGGGCTGGAGGTGGACTG 3` R: 5' CAGAGGCGCAAGTACGTGAG 3`	201 bp	60°C
GP9 Promoter	F: 5' TTCCAAGCTGACAAGTGCCA 3` R: 5' GCTCTCAGAACACCTGCCAT 3`	118 bp	60°C
RPL27	F: 5' GCAAAGCGGTCATCGTAAA 3` R: 5' CTTGTGGGCATGAGGTGAT 3`	190 bp	60°C
PPIA	F:5' CACAAACGGTTCCCAGTTTT 3` R:5' TGTCCACAGTCAGCAATGGT 3`	171 bp	60°C

Table S2. Antibodies used for ChIP-qPCR, including target specificity, host species, provider information, catalog numbers, and amounts applied per immunoprecipitation.

Target Mark	Antibody name and Specifity	Host	Company	Cat. No.	Amount/IP	Notes
H4K20me3	Anti-Histone H4 (tri-methyl K20), ChIP-grade	Rabbit	Abcam	ab9053	2–5 µg	Polyclonal
H4K8ac	Anti-Histone H4 (acetyl K8), ChIP-grade	Rabbit	Abcam	ab15823	2–5 µg	Polyclonal
H3K9ac	Anti-Histone H3 (acetyl K9), ChIP-grade	Rabbit	Abcam	ab10812	2–5 µg	Polyclonal
IgG (Negative control)	Normal Rabbit IgG	Rabbit	Diagenode	Included in kit (Kit Cat# C01010051)	Matched volume	Provided in iDeal ChIP-seq Kit

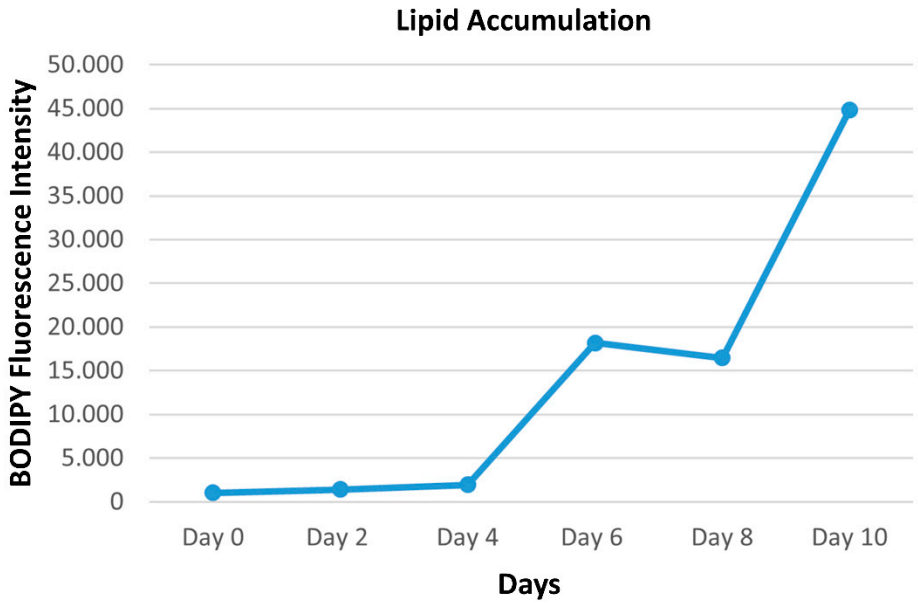

Figure S1. Lipid accumulation during adipogenic differentiation assessed by BODIPY fluorescence intensity. A gradual increase in lipid accumulation was observed over time, with a marked elevation from day 6 onwards and a peak at day 10. Data represent mean fluorescence intensity values corresponding to each time point.

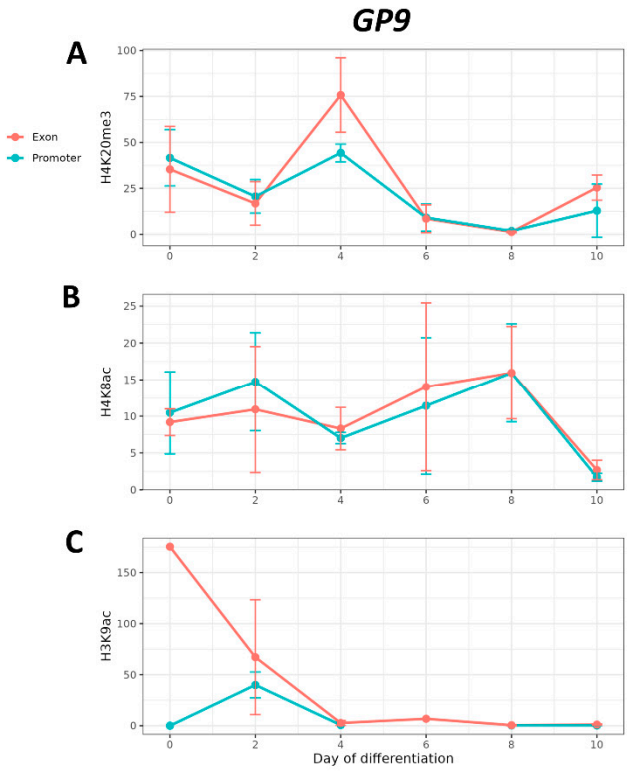

Figure S2. Histone modification profiles at the *GP9* locus during adipogenic differentiation. Enrichment of H4K20me3 (A), H4K8ac (B), and H3K9ac (C) at promoter and exon regions of the *GP9* locus across six differentiation stages (from day 0 to day 10) in porcine mesenchymal stem cells. Histone mark enrichment was determined by ChIP-qPCR and expressed as percentage of input DNA.

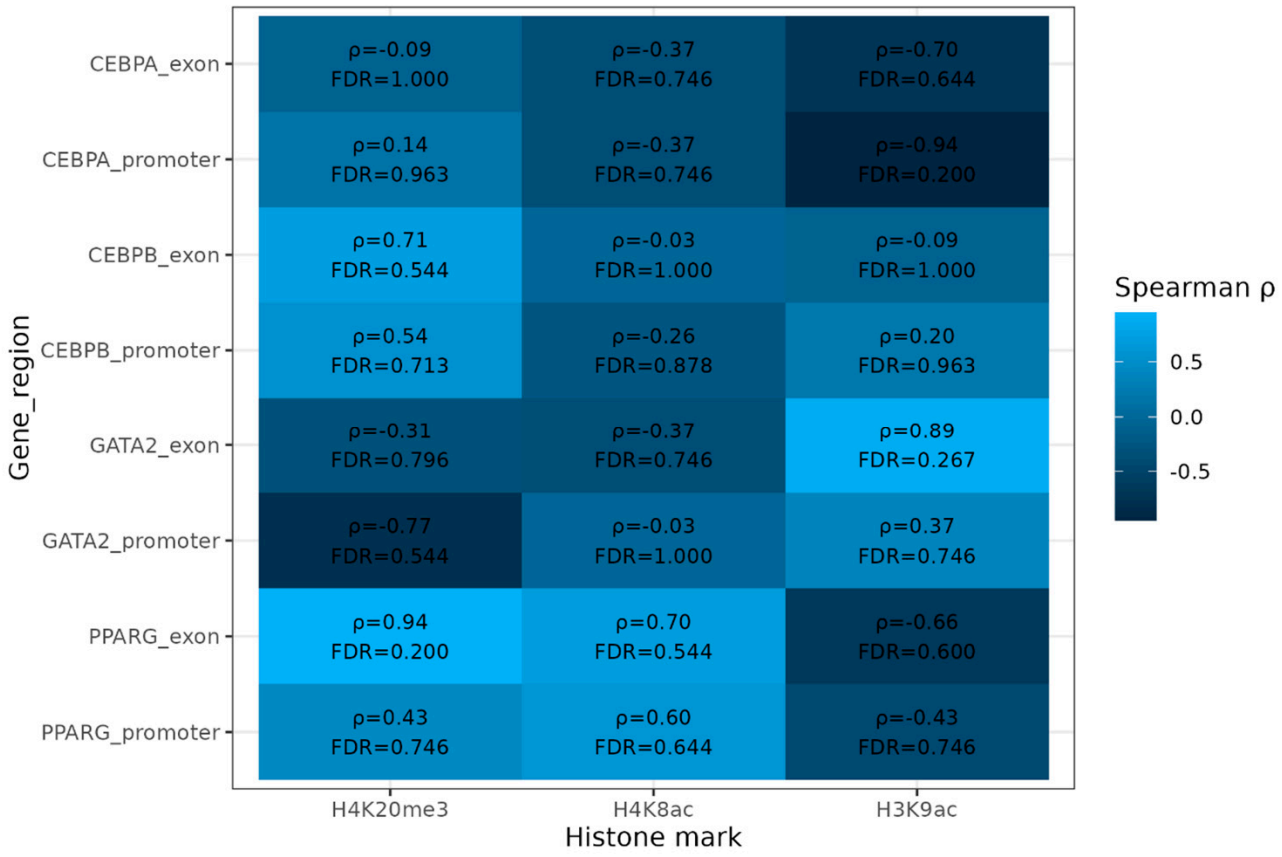

Figure S3. Spearman correlation analysis between histone mark enrichment and gene expression. Heatmap representation of Spearman correlation coefficients (ρ) between histone mark enrichment (H4K20me3, H4K8ac, and H3K9ac) at promoter and exon regions and corresponding gene expression levels across differentiation stages. Correlation coefficients and FDR-adjusted p-values (Benjamini–Hochberg correction) are indicated within each cell. No correlations reached statistical significance after multiple testing correction.

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Statement of the author of the doctoral dissertation

I hereby declare that the submitted doctoral dissertation entitled: **Transcriptional and epigenetic regulation of in vitro adipogenic differentiation of porcine Mesenchymal Stem Cells (MSCs),** was prepared independently by me and that:

— I did not commission the preparation of the dissertation or any of its parts to any other person;
— I did not copy the dissertation or any of its parts from other works related to the subject of my research;
— all elements of the dissertation originating from other sources, including quotations, figures, tables, and scientific data, have been properly acknowledged and cited;
— the dissertation has not previously been submitted in any procedure related to obtaining an academic degree;
— the present printed version of the dissertation is identical to the submitted electronic version.

I am aware that submitting a false statement may result in consequences related to the doctoral degree procedure in accordance with applicable regulations.

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