Negative Elongation Factor A and B are required for normal porcine embryo development

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ABSTRACT

The successful progression of early embryonic development relies on cellular processes such as epigenetic modifications, chromatin reorganization, and the regulation of transcription.

Conserved transcription mechanisms in eukaryotes include RNA Pol II pausing, mainly driven by the Positive Transcription Elongation Factor b (PTEF-b), DRB sensitivity inducing factor (DSIF), and Negative Elongation Factor (NELF) complex. Previous research in mESCs has identified NELF-a participation during the 2-cell-like cell stage, a critical reprogramming event, while NELF-b in the same model has been shown to regulate cell differentiation events. The significance of the NELF complex regulating RNA pausing as development commences has not been reported in porcine embryos. Therefore, this study was designed to evaluate the consequences of attenuating NELF-a and NELF-b mRNAs on early development of porcine embryos.

Specifically, we employed DsiRNAs targeting two distinct regions of NELF-a mRNA (si-NELF-a) or NELF-b mRNA (si-NELF-b) to investigate their roles during early embryo development. The specific objectives of this thesis were to: 1) Evaluate the presence of NELF-a and NELF-b during the early stages of development in the porcine embryo by analyzing mRNA expression; 2) Analyze the impact of NELF-a and NELF-b mRNA attenuation during porcine embryonic development; and 3) Investigate the role of NELF-a and NELF-b in early porcine embryonic development by studying the possible pathways affected by NELF-a and NELF-b attenuation. The findings of our study revealed that NELF-a and NELF-b transcripts are expressed in porcine oocytes and embryos.

Secondly, we found that the attenuation of either NELF-a or NELF-b alone, as well as the simultaneous attenuation of both NELF-a and NELF-b, significantly impaired embryo development, leading to reduced blastocyst formation. Furthermore, we observed that embryos

subjected to NELF-a and NELF-b attenuation exhibited a developmental arrest at earlier stages compared to embryos treated with a control DsiRNA (si-CT). Lastly, we found that NELF-a, NELF-b, or NELF-ab attenuation impaired the normal mRNA levels of genes involved in Embryonic Genome Activation (EGA) regulation. Collectively, these results revealed a critical role of NELF-a and NELF-b in the normal regulation of porcine embryo development.

ABRÉGÉ

La progression normale du développement embryonnaire précoce dépend de plusieurs processus cellulaires tels que les modifications épigénétiques, la réorganisation de la chromatine et la régulation de la transcription. Les mécanismes de transcription conservés chez les eucaryotes comprennent la pause de l'ARN Pol II, principalement dirigée par les complexes PTEF-b, DSIF et NELF. Des recherches antérieures sur les cellules souches embryonnaires de souris mESCs ont identifié la participation de NELF-a lors d'une transition à un stade de différentiation similaire au stade de 2 cellules embryonnaires, ceci étant un événement de reprogrammation cellulaire critique, tandis que NELF-b peut aussi réguler les événements de différenciation cellulaire dans le même modèle. L'importance de la régulation de la pause de l'ARN par le complexe NELF au début du développement n'a pas été étudiée chez l'embryon porcin. Par conséquent, cette étude a été conçue pour évaluer les conséquences de l'atténuation des ARNm de NELF-a et NELF-b sur le développement précoce des embryons porcins. Plus précisément, nous avons utilisé des DsiARN ciblant deux régions distinctes de l'ARNm de NELF-a (si-NELF-a) ou de l'ARNm de NELF-b (si-NELF-b) pour étudier leurs rôles. Ainsi, les objectives spécifiques de cette thèse était de: 1) Évaluer la présence de NELF-a et NELF-b au cours des étapes initiales du développement des embryons porcins en analysant l'expression de l'ARNm. 2) Analyser l'impact de l'atténuation de l'ARNm de NELF-a et NELF-b sur le développement embryonnaire porcin.

3) Étudier les rôles de NELF-a et NELF-b dans le développement embryonnaire précoce en analysant les voies possibles affectées par l'atténuation de l'ARNm de NELF-a et NELF-b.

Les résultats de nos études ont révélé que les transcrits de NELF-a et NELF-b sont exprimés chez l'ovocyte et l'embryon porcin à différents étapes du développement précoce.

Deuxièmement, nous avons constaté que l'atténuation de l'ARNm de NELF-a ou NELF-b individuellement, ainsi que leur atténuation simultanée, ont significativement réduit le développement embryonnaire au stade de blastocyste. De plus, nous avons observé que les embryons soumis à l'atténuation de ARNm de NELF-a et NELF-b présentaient un arrêt du développement à un stade précoce par rapport aux embryons traités avec un ARN d'interférence contrôle (si-CT). Enfin, nous avons constaté que l'atténuation de l'ARNm de NELF-a, NELF-b ou NELF-ab a changé les niveaux d'ARNm des gènes essentiels à la régulation de l'activation du génome embryonnaire (EGA). Dans l'ensemble, nos études ont révélé un rôle critique de NELF-a et NELF-b dans la régulation du développement embryonnaire porcin.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Authors of the presented manuscript:

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Vilceu Bordignon obtained research funds, designed experiments, analyzed data, reviewed the manuscript, and supervised the primary author. María Elena Carrillo Herrera conducted the experiments, analyzed data, and wrote the manuscript. Werner Giehl Glanzner, Mariana Priotto de Macedo, Karina Gutierrez, Luke George Currin, Zigomar da Silva, Vanessa Guay and Hernan Baldassarre assisted in conducting the experiments and analyzing the data.

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LIST OF ACRONYMS

ANOVA: Analysis of Variance.

ARTs: Assisted Reproductive Technologies.

BSA: Bovine serum albumin.

BRCA1: Breast Cancer gene 1.

cAMP: cyclic adenosine monophosphate.

CB: Cytochalasin B.

cGMP: Cyclic guanosine monophosphate.

COC: Cumulus-oocyte complex.

CTD: C-terminal domain.

DAPI: 4',6-diamidino-2-phenylindole.

DNA: Deoxyribonucleic acid.

DRB: 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole.

DSIF: DRB sensitivity inducing factor.

DsiRNA: Dicer-substrate small interfering RNA.

DUX: Double-Homeobox.

EGA: Embryonic Genome Activation.

EGF: Epidermal growth factor.

ESC: Embryonic Stem Cells.

FSH: Follicle Stimulating Hormone.

GTFs: General Transcription Factors.

GVBD: Germinal vesicle breakdown.

hCG: Human Chorionic Gonadotropin.

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ICM: Inner Cell Mass.

ICSI: Intracytoplasmic Sperm Injection.

IDT: Integrated DNA Technologies.

ION: Ionomycin.

IVC: *In vitro* embryo culture.

IVEP: *In vitro* embryo production.

IVF: *In vitro* fertilization.

IVM: *In vitro* maturation.

LH: Luteinizing hormone.

MAPK: Mitogen-activated protein kinase.

MPF: Maturation-Promoting Factor.

mTBM: modified Tris-Buffered Media.

MYC: myelocytomatosis oncogene.

NELF: Negative Elongation Factor.

N-TEF: Negative Transcriptional Elongation Factor.

OAM: Outer acrosomal membrane.

OET: Oocyte-to-embryo transition.

PA: Parthenogenetic Activation.

pFF: Porcine Follicular Fluid.

PIC: Preinitiation complex.

PKA: Protein Kinase A.

PM: Plasma membrane.

P-TEFb: Positive Transcription Elongation Factor b.

PZM-3: Porcine Zygote Medium 3.

Rdbp: RD RNA -binding protein.

RNA: Ribonucleic Acid.

SCNT: Somatic Cell Nuclear Transfer.

si-CT: Dicer-substrate small interfering RNA scrambled sequence.

si-NELF-a: Dicer-substrate small interfering RNA targeting NELF-a.

si-NELF-ab: Dicer-substrate small interfering RNA targeting NELF-a and NELF-b.

si-NELF-b: Dicer-substrate small interfering RNA targeting NELF-b.

SOAF: Sperm/originated oocyte-activating factor.

TCM: Tissue Culture Medium.

Thl1: Trihydrophobin 1-like.

TOP2A: DNA topoisomerase 2a.

TPEN: N,N,N,N-Tetrakis(2-pyridylmethyl)ethylenediamine.

TSS: Transcription Start Site.

WHSC2: Wolf–Hirschhorn Syndrome Candidate 2.

ZP: Zona pellucida.

2CLC: Two-cell like cell.

CHAPTER 1: INTRODUCTION

Assisted Reproductive Technologies (ARTs) have garnered significant attention in the field of reproductive medicine, playing a vital role in understanding fertility issues in both livestock and humans [3]. In a world where the prevalence of infertility has rapidly increased, the use of ARTs has emerged as an exceptional alternative [4, 5], providing valuable options for couples facing challenges in conceiving due to factors such as age, hormonal imbalances, genetic disorders, and other causes [6, 7].

The refinement of ARTs is substantially attributed to advances in reproductive research, opening new pathways for acquiring knowledge to better understand human fertility, genetics, and early embryo development, ultimately leading to improved treatments for reproductive health issues [1].

Examples of such fundamental advancements include the use of *In Vitro* Embryo Production (IVEP) techniques, which are composed of the maturation of the oocytes (IVM), followed by *In vitro* fertilization (IVF), Parthenogenetic Activation (PA) or Intracytoplasmic Sperm Injection (ICSI), and *In Vitro* Embryo Culture (IVC). These techniques serve as excellent models for studying the key players in fertilization and early embryo development. [8, 9].

The first hours after fertilization determine the fate of the embryo and are decisive for the organism's survival in the early stages of development [10]. Embryos that develop correctly meet certain conditions, such as being derived from two healthy and properly matured gametes [11].

The fusion of these two gametes triggers a cascade of molecular processes, reprogramming the embryo cells to a totipotent state. In other words, the embryo reaches the highest level of developmental plasticity, giving rise to cells with the ability to form both the embryonic and

extraembryonic tissues [12, 13]. This action relies on the parental genomes for a short period of time, but for an embryo to continue developing beyond the reprogramming phase, the embryo genome must be expressed. The transcription control of development is attained by the embryo through a process known as Embryonic Genome Activation (EGA) [14].

Previous studies have identified cellular events as important regulators of the EGA, including histone modifications, epigenetic reprogramming, chromatin remodeling, and transcription regulation [15].

Intrinsic interplay of transcription factors is important for the regulation of gene expression during early embryo development, determining in this manner the signaling pathways necessary to delimitate the cell fate, therefore, influencing the different cell lineages and tissue formation.

A better comprehension of this molecular process is crucial for improving reproductive techniques.

Therefore, the focus of this research is to evaluate the roles of NELF-a and NELF-b genes during early stages of embryo development, as they have been identified to participate in cellular reprogramming and differentiation processes in mESCs [16, 17]. However, their role in the regulation of porcine embryo development was still to be investigated. This thesis contains various sections that include the description of background information about early embryo development and *in vitro* embryo production, followed by a manuscript (to be summited for publication in Biology of Reproduction), which contains the methodology, results, discussion, and conclusion, as well as a general discussion about the research findings, and the list of references.

CHAPTER 2: LITERATURE REVIEW

2.1 Importance of swine as a research model

Advances in human medicine often rely on the use of animal models [18, 19]. Mice have served as a mainstay in biomedical research and have been extensively studied as a mammalian model [20]. However, mouse research may not always provide the most suitable model for translational biomedical research [21]. Non-rodent models, such as pigs, can complement our understanding in predictive preclinical studies more effectively [20, 22]. Indeed, pigs have already proven their importance as models for studying several conditions, including cardiovascular diseases [23], diabetes [24], cystic fibrosis [25], Duchenne muscular dystrophy [26], and Alzheimer's disease [27, 28]. Porcine models are now widely used due to their similarities in physiology, anatomical traits, body size, organ size, reproductive cycles, and pathophysiological responses. Consequently, they are valuable species for the development of new therapies for human diseases, stem cell research, and research on fertility and embryo development [28-30]. It is important to highlight that the creation of porcine models relies on embryonic and reproductive technologies along with genomic editing tools [20]. Moreover, pigs are an important part of global food production systems and contribute significantly to food security for humans worldwide as an important source of protein, given their efficiency in converting feed into valuable proteins.

Therefore, studying the fundamental mechanisms that regulate pig embryo development and fertility holds significant implications for enhancing food production and ensuring the long-term sustainability of the agricultural sector [9, 20].

2.2 Transcriptional regulation

The regulation of eukaryotic gene transcription, governed by Pol II, comprises a complex process that involves three major stages: initiation, elongation, and termination [31-33]. The elongation stage is widely regarded as the most critical step in nuclear transcription. Misregulation of this stage can result in various diseases, such as cancer, autoimmunity, neurological disorders, diabetes, cardiovascular diseases, and obesity [34, 35]. This emphasizes the unparalleled importance of comprehending this regulatory mechanism.

Previous studies identify RNA polymerase II promoter-proximal pausing (Pol II pausing) and its release to productive elongation as critical aspects of metazoan gene regulation [36-38]. This mechanism governs the transcription of more than one-third of mammalian protein-coding genes, at least once in the organism's life cycle [39-41]. RNA Pol II pausing refers to the disproportional accumulation of RNA pol II downstream of the transcription start site (TSS) in both Drosophila [42-45] and mammalian cells [40, 46–48]. During this process, RNA Pol II remains active and engaged on the DNA template until it receives signals to commence elongation [36].

The process of regulating eukaryotic transcription for every active gene starts with the preinitiation complex (PIC) [49]. This involves the orchestration of RNA Pol II, general transcription factors (GTFs), chromatin remodeling complexes, and cell signaling pathways, along with other molecular events, all converging at the +1 TSS [32, 46, 50]. Subsequently, following the transcription of approximately 20-50 base pairs downstream by the activated RNA Pol II (distinguished by Ser5 phosphorylation in the CTD), transcriptional pausing takes place. During this pausing, NELF and DSIF play crucial roles by binding to the nascent transcript [46, 51].

2.2.1 Mechanism of RNA Polymerase II Pause-Release

It has been reported that approximately 60% of RNA Pol II stalling in mammals is driven by the mechanism of Promotor Proximal Pausing (PPP), which was first identified in 1985 in Drosophila heat-shock genes [43, 52, 53]. RNA Pol II pausing near the promoters is induced by the action of positive and negative regulators, including the Negative Transcriptional Elongation Factor (N -TEF) [54], which is composed of the NELF and the DSIF. In addition, N-TEF reduces elongation rates and enhances the stability of RNA Pol II binding to the DNA template [55, 56].

The pausing-release mechanism that unleashes RNA Pol II for productive elongation is orchestrated by the recruitment of the positive transcription elongation factor b (P-TEFb) [57-58].

Subsequently, this complex allows the generation of nascent RNA by phosphorylating Ser2 within the C-terminal domain (CTD) of RNA Pol II's largest subunit, DSIF and NELF [59-63].

This phosphorylation event triggers the dissociation of the NELF complex, enabling the recruitment of elongation factors [59]. Consequently, the DSIF complex assumes an elongation-stimulating role [46, 64].

Negative Elongation Factor Complex

As described above, NELF plays an indispensable role in mammalian transcription in conjunction with DSIF and RNA Pol II [1,40]. N-TEF negatively influences transcription regulation by causing RNA Pol II to spend more time in pausing sites [65]. NELF is a multiprotein complex composed of four subunits, namely, NELF-a (or WHSC2, Wolf- Hirschhorn syndrome candidate 2), NELF-b (or Cobra1, Cofactor of BRCA1), NELFc/d (or Thl1, Trihydrophobin 1-like), and NELF-e (or Rdbp, RD RNA -binding protein) [57]. Previous studies have characterized this complex as a highly interdependent and coordinated complex when it comes to participating

in gene transcription [66, 67]. There is evidence that the NELF complex is critical for biomolecular processes, including the heat shock response [61], estrogen signaling [68], IL-6 signaling [53], the inflammatory response [69], FGF/ERK signaling [70], cell growth [70, 71], and regulation of early embryonic development [17, 70]. This underscores the biological significance of the NELF complex [67].

Previous research has noted that both early embryogenesis and cancer progression share common characteristics, such as rapid cell proliferation [72]. Furthermore, a recent study examined the profile of NELF-regulated genes in human breast cancer cell line T47D following the knockdown of each NELF subunit. The gene ontology analysis revealed the involvement of NELF in the regulation of the cell cycle, cell cycle processes, cell division, cellular component organization, and biogenesis, among other biological events [73]. Despite these prior efforts to comprehend the functions of the NELF complex and promotor-proximal pausing in a developmental context, many aspects remain poorly understood [40]. Additionally, the roles of each NELF subunit have been investigated and are highlighted below.

NELF-a

The member A of the Negative Elongation Factor complex is encoded by the WHSC2 gene and shares sequence homology with the hepatitis delta antigen, a viral protein that binds and activates RNA Pol II [66, 74]. NELF-a has been found to participate in several biological processes [16]. Recent studies indicate that NELF-a triggers a delay in the progression from the S phase to the M phase of the cell cycle, reduces DNA replication, and alters chromatin assembly when there is haploinsufficiency in human lymphoblastoid cells [75]. On the other hand, it has also been shown that acute NELF-a knockdown restricts compensatory gene expression and leads to

ventricular dysfunction in cardiomyocytes during cardiac hypertrophy in a mouse model [76]. Additionally, inhibiting NELF-a in heat-shock protein (HSP) cells contributes to the maintenance of genome integrity [77]. Interestingly, Hu et al. identified NELF-a as one of the earliest drivers of the 2 cell-like state in embryonic stem cells (ESCs) [16] Moreover, NELF-a collaborates with DNA topoisomerase 2a (TOP2A) in a specific interaction that promotes the activation of Double-Homeobox (Dux), a key regulator of the 2 cell-like state [78].

NELF-b

Negative Elongation Factor subunit B (NELF-b), also known as Cobra1, is a crucial component of the NELF complex [17], and it serves as a precursor to the tumor suppressor gene product Breast Cancer gene 1 (BRCA1) [79, 80]. Importantly, studies by Abuhashem et al. have established that NELF-b is indispensable for the early stages of mice pre-implantation development, specifically during the zygote-to-2-cell transition [81]. Moreover, prior research has demonstrated that disrupting Cobra1 in a mouse model results in an inner cell mass (ICM) deficiency and leads to embryonic lethality at the time of implantation. [17].

NELF-cd

Negative Elongation Factor subunit CD (NELF-cd), also known as Thl1, has been found to be overexpressed when examined in colorectal cancer cells (CRC). This increased expression has been associated with a potential therapeutic approach for CRC treatment, indicating a significant role in elucidating oncogenic processes in tumors with elevated NELF levels [82, 83].

However, the specific mechanism behind NELF-cd's oncogenic role in CRC remains unclear, necessitating further research.

NELF-e

Negative Elongation Factor subunit E (NELF-e), also known as Rdbp, possesses RNA-binding activity that is recognized as critical for its inhibitory role in NELF's function for transcription regulation [54, 74]. NELF-e has been identified as a facilitator of chromatin accessibility through SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily B, Member 1 (SMARCB1), which is required for myelocytomatosis oncogene (MYC)-induced transcription [84]. Furthermore, NELF-e promotes gastric cancer growth and metastasis through the transcription factor E2F2 (E2F2) in gastric cancer tissues [85]. Studies conducted by Dang et al. suggested that NELF-e may contribute to transcriptome imbalance in hepatocellular carcinoma (HCC) by regulating MYC signaling [86].

2.3 Oocyte maturation and early embryo development

2.3.1 Oocyte maturation

Oogenesis is a specialized process that starts with the initial oocyte in the dormant primordial follicle and culminates in the production of a mature oocyte [4, 87, 88]. The duration of this process varies among species, taking only weeks in mice, but several months in pigs and humans. Oocyte maturation involves two main components, nuclear or meiotic maturation and cytoplasmic maturation [89, 90]. In the later stages of oogenesis in post-pubertal ages, mammalian oocytes within antral follicles remain arrested at the prophase I of meiosis, but are induced to continue their meiotic maturation (resumption of the meiosis) by the release of the gonadotropin Luteinizing Hormone (LH), which also induces ovulation [91]. Is during the later growth inside the preovulatory follicles that oocytes acquire both meiotic and developmental competence [4, 92].

Oocyte maturation is tightly regulated, primarily through cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). While cAMP maintains oocytes in the prophase I stage [93], cGMP, which is transferred from the cumulus cells to the oocyte via gap junctions, prevents cAMP degradation by inhibiting activation of the oocyte phosphodiesterase PDE3A [94, 95]. The LH surge triggers oocyte maturation by reducing cGMP production, closing cell gap junctions, via mitogen-activated protein kinase (MAPK), and lowering cAMP levels in the oocyte [90, 96]. The decrease in cAMP leads to reduced protein kinase A (PKA) activity, resulting in the activation of the Maturation-Promoting Factor (MPF) through dephosphorylation by the M-phase inducer phosphatase 2 (CDC25B) [97].

Structurally, the chronological changes observed during nuclear maturation include germinal vesicle breakdown (GVBD), chromatin condensation, metaphase I, anaphase I, telophase I, and metaphase II, when the oocyte extrudes the first polar body and remains arrested at this stage until fertilization [89]. Cytoplasmic maturation involves organelle modification and redistribution, dynamic changes in the cytoskeleton, and storage of molecules (e.g., RNA, proteins). In addition, organelles change in number, size, and location, which depends on cytoskeleton rearrangements [98, 99]. In general, the final stage of oocyte maturation can be described as the stage during which transcription diminishes and stored components from oogenesis start to be removed in preparation for subsequent steps of development, including fertilization, completion of the meiotic maturation, formation of the maternal and paternal pronuclei, first cleavage, and early embryo development [100].

2.3.2 Fertilization

In most invertebrates (such as sea urchins) and nonmammalian vertebrates (like fish and amphibians), spermatozoa are fully capable of fertilizing eggs as soon as they are produced in the testes [101]. However, in mammals, including humans and pigs, sperm cells produced in the testes are not initially capable of fertilization. Instead, their ability to fertilize oocytes develops following sperm capacitation and the acrosome reaction [102, 103]. During sperm capacitation, which occurs in the female reproductive tract [104], sperm undergo various biochemical, physiological, and cellular modifications aimed at enabling sperm to bind to the zona pellucida (ZP) and go through acrosome exocytosis [105-107]. Consequently, the sperm membrane becomes more fluid, exposing surface proteins necessary for interactions with the oocyte [108]. This process involves a reduction in intracellular sodium (Na+), activation of potassium (K+) channels, pH elevation, and the increase in cyclic AMP (cAMP) levels, leading to the activation of protein kinase A (PKA) and tyrosine phosphorylation [109]. These changes trigger sperm hyperactivated motility, zona pellucida binding, and prepare for acrosome exocytosis, which is crucial for fertilization [110, 111]. Once capacitated, sperm are attracted to the oocyte by soluble extracts found in the follicular fluid. Subsequently, the sperm attaches to glycoproteins on the zona pellucida (ZP) of the oocyte. In the case of porcine and bovine oocytes, the critical sperm-binding activity is attributed to the ZP3/ZP4 complex [112–114].

The binding of sperm to ZP-glycoproteins has several effects on sperm, including G protein activation, membrane depolarization, Ca2+ channel activation, and increased intracellular Ca2+ and pH levels [115]. Consequently, the fusion of the sperm plasma membrane (PM) with the sperm outer acrosomal membrane (OAM) releases acrosomal contents [116]. Sperm motility is crucial for penetrating the ZP [117]. Acrosin enzymatic activity drives the degradation of the ZP and alters

the acrosome structure, exposing the inner acrosomal membrane, and priming the sperm for binding and fusion [118]. Once crossing the ZP, the sperm binds to the oocyte plasma membrane (oolemma) [117, 119].

Upon fusing with the oocyte, the sperm introduces a sperm-originated oocyte-activating factor (SOAF) to initiate oocyte activation [120]. SOAF triggers an intracellular increase in Ca2+, a crucial signaling mechanism for oocyte activation [121, 122]. Following fusion, cortical granules are released from the oocyte into the perivitelline space [123, 124], preventing polyspermic fertilization [125–128]. As a result, the oocyte completes its meiosis, by extruding the second polar body, and initiates the early stages of embryo development.

2.3.3 Blastocyst formation

Once meiosis is completed, the embryo undergoes multiple mitotic divisions and cell cleavages, ultimately reaching the blastocyst stage. During this stage of development, the embryonic cells are called blastomeres [129-131]. After several cell divisions, the embryo undergoes compaction, during which blastomeres increase their contact and adhesion, forming a cell mass named morula [132]. E-Cadherin (CDH1), which depends on extracellular calcium, plays a vital role in cell adhesion and morula compaction [133]. The blastocyst's fluid-filled cavity, known as the blastocoel, forms with the assistance of Na/K-ATPase in the trophoblast, which refers to the outer layer of cells that will ultimately give rise to the extraembryonic tissues [134–136].

Some blastomeres cluster to create the inner cell mass, located inside the blastocyst, which later differentiate into the various cell types forming the different tissues and organs of the fetus.

As fluid accumulates in the blastocoel and cell multiplication continues, the embryo expands, gradually thinning the ZP until the blastocyst eventually hatches from it. During hatching,

the embryo is located within the uterine lumen, and the trophoblast is responsible for implanting the embryo into the uterine wall [130].

2.3.4 Early embryonic development

Embryo development represents a critical step following fertilization which determines the survival and preservation of living organisms [137, 138]. To better understand this biological process, it is necessary to analyze the factors and key steps involved in normal embryo development [139].

Mammalian embryonic development relies on a sequence of biological events starting with the fusion of two healthy and fully mature gametes, following by the acquisition of cellular totipotency, activation of transcriptional activity, cell differentiation, and lineage specification [140–142]. The successful completion of these steps is fundamental for normal fetal and offspring development, and it represents a crucial research area to improve the efficiency of assisted reproductive technologies (ARTs) through a better understanding of the biological mechanisms governing early embryo development [143, 144].

The exploration of early developmental stages began in the early 1800s when pioneers like Van der Stricht, Von Baer, Bischoff, and others analyzed canine oocytes and embryos [145-148].

Building upon these foundational studies, it is now understood that mammalian preimplantation development encompasses a series of conserved evolutionary events ranging from zygote formation to the blastocyst stage [12]. However, variations in the regulation of events between different species have emerged [20, 149], adding complexity to the preimplantation period and highlighting its vulnerability in mammalian development [150, 151]. Moreover, the growing interest in the use of ARTs and gene editing in animal models have further raised the interest in

achieving a more systematic comprehension and elucidation of the regulatory mechanisms governing early embryogenesis [152-153].

2.4 Embryonic Genome Activation

Following fertilization, newly formed embryos undergo a significant developmental transition characterized by an initial transcriptional quiescence of the genome. This state triggers a reprogramming phase towards totipotency [154-156]. This temporary transcriptional inactivity allows the embryo to navigate subsequent phases involving substantial epigenetic remodeling and parental genome reorganization [155, 157-159]. EGA signifies the genetic shift from oocyte to embryo, dictating development as it involves the degradation of maternally derived RNAs and proteins enabling the embryo to advance to subsequent stages of development in a seamless progression (Figure 1) [160-163]. EGA facilitates subsequent development by regulating transcription through epigenetic reprogramming and activation of essential transcription factors, leading to cell differentiation and lineage specification [129, 164-168]. In a developmental context, cell specification depends on precise changes that impact gene expression, primarily governed by transcriptional regulatory mechanisms and post-transcriptional modifications [10, 169–171].

Furthermore, the pivotal stage of EGA seems to be linked to crucial events that trigger the onset of cell differentiation [172-173]. This phase varies in its timing during embryonic development across different species [174]. In bovine, it occurs during the transition from the 8-16 cell stage, in sheep at the 8-cell stage, and in pigs and human embryos at the 4-8 cell stage (Figure 1) [20]. These similarities among preimplantation development models position the pig as a representative model for investigation of the EGA transition [175].

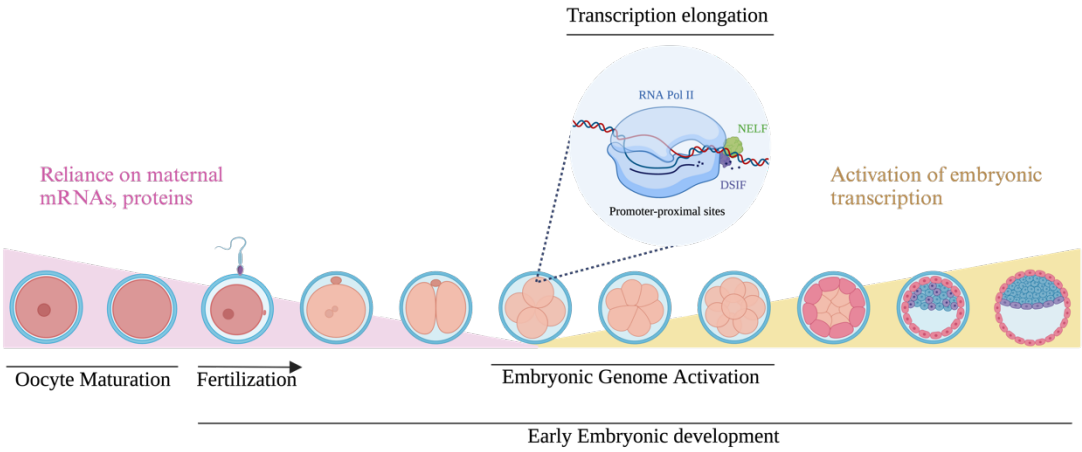

Figure 1. Regulation of transcription at early stages of embryonic development via RNA Polymerase II (RNA Pol II) Pausing. Transcription elongation is regulated by the association of DSIF and the NELF complex. During early embryonic stages of mammalian development, Pol II pauses at the promoter-proximal sites [1,2].

Transcription factors (TFs) and the recruitment of the RNA Pol II to the promoter region of genes are essential for proper gene transcription and, consequently, synthesis of proteins required for normal EGA and embryo development [167, 168, 176, 177]. In light of the crucial role of transcription regulation during EGA and the participation of NELF-a and NELF-b in cellular reprogramming and differentiation events, we hypothesize that the NELF-a and NELF-b subunits of the NELF complex serve as key elements driving normal embryo development. Our investigation aims to elucidate their specific roles and contributions in orchestrating the intricate processes of embryogenesis, ultimately advancing our understanding of early developmental pathways.

CHAPTER 3: RATIONAL, HYPOTHESIS AND OBJECTIVES

Comprehending the intricacies of transcriptional regulation at the onset of development is crucial for understanding how an organism transitions from a reprogramming state to the complex process of cellular differentiation [178, 179]. EGA represents the pivotal moment when embryonic transcription initiates during early embryo development [180]. Within this complex regulatory landscape, RNA polymerase pausing emerges as a fundamental mechanism in mammalian cells.

Yet, the precise mechanisms governing RNA polymerase II (Pol II) and its transition into productive elongation during the EGA phase remain elusive.

As it has been demonstrated, NELF-a and NELF-b are key factors involved in the regulation of mammalian transcription, and cell reprogramming and differentiation in mESCs.

Therefore, we hypothesized that NELF-a and NELF-b play essential roles in EGA and regulation of early embryonic development.

To explore this hypothesis, the following objectives were addressed in this study:

1. Evaluate the presence of NELF-a and NELF-b during early stages of development in the porcine embryo model by the analysis of mRNA expression.
2. Analyze the impact of NELF-a and NELF-b attenuation during porcine embryonic development via interference RNA.
3.Characterize the role of NELF-a and NELF-b on early procine embryonic development by studying the possible pathways affected by NELF-a and NELF-b attenuation.

CHAPTER 4: ARTICLE

Manuscript will be submitted for publication to Biology of Reproduction

Both NELF-a and NELF-b are required for normal development of porcine embryos

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Short title: NELF-a and NELF-b regulate embryo development

4.1 Abstract

Embryonic genome activation (EGA) and the maintenance of cell pluripotency are critical events in normal embryo development [1, 2]. However, the mechanisms governing these processes remain incompletely understood, especially in the context of livestock embryos. The Negative Elongation Factor (NELF), a transcription factor complex, exerts a critical role by negatively modulating transcription elongation via RNA polymerase II pausing [3]. Previous research has signaled the involvement of NELF-a and NELF-b in the control of cell reprogramming and differentiation [4, 5]. Although NELF-a and NELF-b were found to be transiently expressed during EGA in mouse embryos, their specific functions in the regulation of early development have not been elucidated. Therefore, this study was designed to evaluate the consequences of attenuating NELF-a and NELF-b mRNAs on early development of porcine embryos. Specifically, we employed DsiRNAs targeting two distinct regions of NELF-a mRNA (si-NELF-a) or NELF-b mRNA (si-NELF-b). These DsiRNAs were microinjected into the cytoplasm of *in vitro* matured and parthenogenetically activated oocytes, which were subsequently cultured for a duration of 7 days *in vitro* to evaluate blastocyst rates and total cell numbers as indicators of embryonic development. The findings of our study revealed that the attenuation of either NELF-a or NELF-b alone, as well as the simultaneous attenuation of both NELF-a and NELF-b, significantly impaired embryo development to the blastocyst stage. Furthermore, we observed that embryos subjected to NELF-a and NELF-b attenuation exhibited a developmental arrest at an earlier stage compared to embryos treated with a control siRNA (si-CT). Additionally, we found that NELF-a, NELF-b, and NELF-ab attenuation impaired the normal mRNA levels of essential genes of EGA regulation.

These results revealed a critical role of NELF-a and NELF-b in the normal regulation of porcine embryo development.

4.2 Introduction

Fertility issues in livestock and the human population have been increasing over the last decades. Strategies to counteract this situation are required for the preservation of species as well as animal conservation [6]. Recent technologies have been implemented to address this biological situation, just as Assisted Reproductive Technologies (ARTs) which are based on a further understanding of early embryo development [7]. Improvement in these procedures could mitigate some fertility issues by providing baseline information essential for the improvement of the embryo's capabilities to develop normally. Recent studies in pigs, suggested the importance of the regulation of early stages of development in embryos due to unfavorable statistics on *in vitro* procedures as well as in vivo processes where 60% and 25% of embryos fail to reach the final stages of development, respectively [8, 9], highlighting the importance of this early stages on the fate of the embryo.

Early embryo development depends on cellular processes involving epigenetic modifications, alterations in chromatin structure, and the availability and expression of specific transcription factors such as DUX, Myc, Zscan4, Dppa2-4, and NELF [10–14]. Previous research has delved into the roles of these genes in EGA, the stage where developmental control shifts to the embryo [15]. This pivotal phase marks the moment when the embryo's genome encodes transcripts regulating its own development. Understanding the fundamental mechanisms governing transcriptional regulation as development commences is crucial for uncovering the pathways that govern normal embryonic development [16]. In this context, genes participating in EGA regulation and possessing regulatory functions in the transcription of highly active genes [17], like NELF, emerge as potential candidates for establishing normal embryonic development [4].

The goal of this study was to explore the roles of NELF-a and NELF-b during porcine embryonic development. The specific objectives encompass evaluating the presence of NELF-a and NELF-b in the early stages of porcine embryo development through mRNA expression analysis. Additionally, the study aimed to assess the impact of NELF-a and NELF-b attenuation on porcine embryonic development using interference RNA techniques.

4.3 Material and methods

4.3.1 Chemicals

Unless stated otherwise, chemicals and reagents were purchased from Millipore Sigma (Sigma-Aldrich; Oakville, ON, Canada).

4.3.2 Oocyte collection and *in-vitro* maturation

Ovaries from prepubertal gilts were collected from a local abattoir (CBCo Alliance, Les Cèdres, Quebec, Canada) and transported to the laboratory in saline solution (0.9 % NaCl), containing penicillin (100 IU/mL) and streptomycin (10 mg/mL) at 32°C. Cumulus-oocyte complex (COCs) were aspirated from follicles with diameter ranging from 3 to 6 mm using a 21 G needle. Follicle aspirate was centrifuged at 300 rpm for 3 min. COCs were washed in manipulation media (TCM-199 HEPES-buffered medium supplemented with 1% pFF), and those with at least 3 layers of cumulus cells and homogeneous granulated cytoplasm were selected for *in vitro* maturation (IVM). Groups of 30 COCs were matured for 22 h in 90 uL drops of maturation medium covered with mineral oil, in an incubator with an atmosphere of 5% CO2 in air, at 38.5°C.

Maturation medium consisted of TCM199 (Life technologies, Burlington, ON, Canada), supplemented with 0.5 UI/mL hCG (Chorulon®; Merck Animal Health, Kirkland, Quebec,

Canada), 10 µg Armour std./mL FSH (Folltropin-V®; Vétoquinol, Lavaltrie, Quebec, Canada), 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP), 10 ng/mL of epidermal growth factor (EGF; Life technologies), 100 µg/mL cysteine, 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 20 µg/mL gentamicin (Life technologies), and 20% v/v pFF. Following an initial 22-hour culture period, the cumulus-oocyte complexes (COCs) were rinsed and transferred to a new drop of IVM media, which was not supplemented with dbcAMP, LH and FSH, and cultured for an additional 22-24 h.

4.3.2.1 *In Vitro* fertilization (IVF)

Following 44 h of *in vitro* maturation, IVF procedure initiated with the removal of cumulus cells of matured oocytes by vortexing in TCM-199 HEPES-buffered medium (Life Technologies) supplemented with 0.1% hyaluronidase. Denuded oocytes were rinsed three times in modified Tris-Buffered Medium (mTBM) [18], containing 2 mM caffeine and 0.2% bovine serum albumin (BSA, fatty acid free). Fresh boar semen, donated by the Centre d'Insémination Porcine du Québec (CIPQ, Roxton Falls, Quebec, Canada), underwent a washing process in mTBM without caffeine prior to being resuspended in mTBM containing caffeine. Sperm concentration was measured, and motility was evaluated.

Oocytes were then fertilized in groups of 60-80 using $2x10^5$ motile sperm/ml in four-well NUNC plates with 500 µL of mTBM for 5 h.

Presumptive zygotes underwent a final wash with mTBM medium to eliminate any remaining sperm attached to the zona pellucida.

4.3.2.2 Parthenogenetic activation

Following 44 h of *in vitro* maturation, PA procedure initiated with the removal of cumulus cells of matured oocytes by vortexing in TCM-199 HEPES-buffered medium (Life Technologies) supplemented with 0.1% hyaluronidase. Selected matured oocytes with the first polar body were activated using the IT-20 protocol [19]. Briefly, oocytes were exposed to 15 μM ionomycin for 5 min in TCM-199 supplemented with 2 mg/mL of BSA, washed twice in the same medium without ionomycin, following by15 min exposure to 200 μM N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN). Oocytes were then incubated in porcine zygote medium (PZM-3) [20] supplemented with 7.5 μg/mL cytochalasin B and 3 mg/mL BSA for 4 h to prevent the extrusion of the second polar body.

4.3.2.3 *In vitro* culture (IVC)

Presumptive zygotes were cultured in 60 μL drops of PZM-3 medium supplemented with 3 mg/mL BSA, under mineral oil, in a humidified atmosphere of 5% CO_2 and 95% air at 38.5 °C.

On day 5, the culture medium was supplemented with 10% fetal bovine serum (FBS). Embryo cleavage rates were evaluated 48 h (day 2) following IVF or PA, while blastocyst rates were assessed after 168h (day 7) of culture.

4.3.3 NELF-a and NELF-b attenuation

Attenuation of *NELF-a* and *NELF-b* mRNAs were performed to investigate their potential roles during early embryo development. Dicer-substrate interfering RNAs (DsiRNAs) were designed (Custom DsiRNA Design Tool) and synthetized by Integrated DNA Technologies (IDT; Windsor, ON, Canada). Specificity was confirmed by using the Basic Local Alignment Search

Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD, United States). *In vitro* matured and parthenogenetically activated oocytes were microinjected, using an inverted microscope (Nikon, Tokyo, Japan) equipped with a micromanipulator system (Narishige International, Long Island, NY, USA) and a FemtoJet 4i microinjector (Eppendorf, Hamburg, Germany), with 10 pL of 25 µM diluted sense and antisense DsiRNAs targeting two specific sequences in the mRNA of *NELF-a* (si-NELF-a), *NELF-b* (si-NELF-b), both *NELF-a* and *NELF-b* (si-NELF-a + b), or control scrambled sequences (si-CT) (Table 1). Microinjections were performed in TCM-199 HEPES-buffered medium supplemented with 2 mg/ml BSA and 20 µg/ml gentamicin. Knockdown efficiency was evaluated by determining the relative mRNA abundance of *NELF-a* and *NELF-b* by real-time quantitative PCR (qPCR) on day 2 and day 3 of culture after microinjection of DsiRNAs. The experiment was repeated 4 times. The number of embryos used was 262, 243, 267, and 219 for si-CT, si-NELF-a, si-NELF-b and si-NELF-ab respectively.

4.3.4 RNA Extraction and Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA was extracted from pools of 10–15 embryos on day 2 and day 3 of development using the PicoPure RNA isolation kit (Thermo Fisher Scientific). Following extraction, RNA was treated with DNase I (Qiagen; Louiville, KY, United States) to eliminate any potential contamination of genomic DNA, and then reverse transcribed using the SuperScript VILO cDNA synthesis kit (Life Technologies). RT-qPCR reactions were performed in a CFX 384 real-time PCR system (BioRad, Hercules, CA, USA) using the advanced qPCR mastermix (Wisent Bioproducts, St-Bruno, QC, Canada). Primers were designed by using the Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer), based on porcine sequences available in GenBank, and were synthesized by IDT (Table 2). The genes evaluated during early embryo development

consisted of each subunit of the NELF complex (*NELF-a, NELF-b, NELF-cd,* and *NELF-e), SPT5, SPT6,* and *CDK9*. Alternatively, when analyzing *NELF-a*, *NELF-b* and *NELF-ab* attenuation, the genes evaluated consisted of the NELF remaining subunits (*NELF-cd* and *NELF-e*) and selected EGA markers (*DUX, DPPA2, KDM5B,* and *EIF1AX*). The relative abundance of mRNA for each gene was determined by using the standard curve method. Relative mRNA expression was normalized to the mean abundance of the internal control gene *H2A*. Samples were run in triplicates. All reactions had efficiency between 90 and 110%, a coefficient of determination (r^2) >0.98 and slope values from −3.6 to −3. Dissociation curve analyses were performed to validate the specificity of the amplified products. Embryo samples were collected from four individual replicates, each containing 15 embryos per treatment and replicate. For the attenuation experiments, embryo samples were collected from three individual replicates, each containing 11 to 15 embryos per treatment and replicate.

4.3.5 Immunofluorescence and embryo cell counting

Embryos that developed to the blastocyst stage after 7 days of culture were fixed in 4% paraformaldehyde for 15 min and then permeabilized in PBS containing 0.3% BSA and 0.1% Triton X-100 at 4 °C. Before staining, embryos were incubated at 37 °C for 1 h in blocking solution (PBS supplemented with 3% BSA and 0.2% Tween-20). Samples were exposed to 10 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies) in blocking solution for 20 min, rinsed in blocking solution, and then mounted on slides using Mowiol 40-88 mounting solution. The number of cells per blastocyst was determined by counting the number of nuclei in each embryo using an epifluorescence microscope. Total cell numbers per blastocyst were counted from 80, 10, 9, and 9 blastocysts obtained from 8 replicates, for si-CT, si-NELF-a, si-NELF-b and si-NELF-ab

treatments, respectively. A total of 41, 36, 46, and 32 embryos from 4 replicates were used to determine the number of cells in embryos that cleaved but did not reach the blastocyst stage (arrested embryos), for the treatments si-CT, si-NELF-a, si-NELF-b and si-NELF-ab, respectively.

4.3.6 Statistical Analysis

Data was analyzed using the JMP software (SAS institute Inc., Cary, NC). Normality of data was tested using the Shapiro-Wilk W test and normalized when necessary. Statistical differences in the relative mRNA levels Means analyzed by ANOVA following by Student's T test or LSMeans Student's T test for single or multiple comparisons, respectively. Embryo developmental data (cleavage and blastocyst rates), average number of cells per embryo were analyzed by ANOVA following by Tukey-Kramer HSD test. Differences in the proportion of embryos that cleaved but did not reach blastocyst stage were analyzed using the Chi-Square Test in a contingency table. Results are presented as means ± SEM, and $P < 0.05$ was considered statistically significant.

All the experiments were repeated at least three times.

Table 1: List of DsiRNAs used for knockdown experiments

Target	Sense	Antisense
NELF-a #1	rCrArCrUrUrUrCrArGrUrUrGrArArArArGrGrArArGrCrCGA	rUrCrGrGrCrUrUrCrCrUrUrUrUrCrArArCrUrGrArArArGrUrGrUrU
NELF-a #2	rGrArUrUrCrArArUrCrUrUrArCrUrUrUrUrUrUrGrArArUGT	rArCrArUrUrCrArArArArArArGrUrArArGrArUrUrGrArArUrCrCrA
NELF-b #1	rArGrCrUrUrUrUrCrUrCrUrGrGrUrGrArArGrArUrGrCrCGT	rArCrGrGrCrArUrCrUrUrCrArCrCrArGrArGrArArArArGrCrUrCrU
NELF-b #2	rCrGrUrCrArUrGrArArArCrArCrUrUrGrCrCrCrArArGrGTG	rCrArCrCrUrUrGrGrGrCrArArGrUrGrr.UrUrCrArUrGrArCrGrCrA
Negative.Control	rCrUrUrCrCrUrCrUrCrUrUrUrCrUrCrUrCrCrCrUrUrGrUGA	rUrCrArCrArArGrGrGrArGrArGrArArArGrArGrArGrGrArArGrGrA

Table.2 Primers used for quantitative real-time PCR

Target	Forward	Reverse	Accession num. or Reference
NELF-a	GAAAAGGAAGCCGAAGAGCG	TGCTTCGGGATGCCTTTGAG	XM_003128808.5
NELF-b	CAGGGGTTTCTTGACGGAGT	GTCGCACAGGATCATGGACA	NM_001310162.1
NELF-cd	GTCCACATCGAAAGCCGTTG	CCACCACGGGAAACCTGATG.	XM_005673063.3[a]
NELF-e	AAGACATGACACCCACGCTTC	TAGGTGACGAAGGCACAGTTTCTG	XM_005665804.3
CDK9	CTGTCGAACCAAAGCTTCCC	CAGCGTGAACTTGACTAAGACG	NM_001166044.1
SPT5	ACGGTGTACGGAGGATCTGA	CCGCTCCTCCCCAATCTTAC	XM_005655822.3
SPT6	GAGCGAGTCAAGGTGGGAAT	TCCACTCGTTGTTCCTGTCC	XM_021067480.1
DUXA	AGAACACAGACGCAAGCCAA	TAGCTGGTCCGACATCGTCT	XM_021097581.1
EIF1AX	ACACCTCCCCGATAGGAGTC	TTGAGCACACTCTTGCCCAT	NM_001243218.1
DPPA2	TGAGTACCAGTGGCCAGAAAA	GACTGCAATCTGGTCTCCCA	XM_003358822.4
KDM5B	GACGTGTGCCAGTTTTGGAC.	TCGAGGACACAGCACCTCTA	[10]
H2A	AGACTCGCTCTTCTAGGGCT	CGGTCAGATACTCCAGCACC	XM_001927727.2

a Homologous region between 2 transcripts XM_005673063.3 ; XM_013985518.2

4.3.7 Results

Transcripts of members of the NELF complex are expressed in porcine embryos

In this experiment, q-PCR analyses revealed that mRNAs of genes encoding members of the NELF complex or other proteins having functional associations with the NELF complex are expressed in porcine oocytes and embryos. Our results revealed that the relative abundance of transcripts for both NELF members and associated factors fluctuates at different developmental stages. For the NELF complex members, *NELF-a* mRNA decreased in blastocysts compared to day 3 embryos, *NELF-b* mRNA was higher in day 4 compared to day 2 and day 3 embryos, and *NELF-cd* and *NELF-e* mRNAs increased in blastocysts compared to earlier embryonic stages (Figure 1). Transcript levels of the NELF-associated factors, *SPT5*, *SPT6* and *CDK9*, showed a similar profile characterized by a steadily but not statistically significant increase from oocytes to day 4 embryos, followed by a significant decrease at the blastocyst stage (Figure 1). The fluctuation observed in the relative abundance of these transcripts at different embryonic stages suggests they might be involved in the regulation of various developmental functions. To gain insights into the specific functions of the NELF members in the regulation of early embryo development, we decided to assess the impact of attenuating *NELF-a* and *NELF-b* mRNAs.

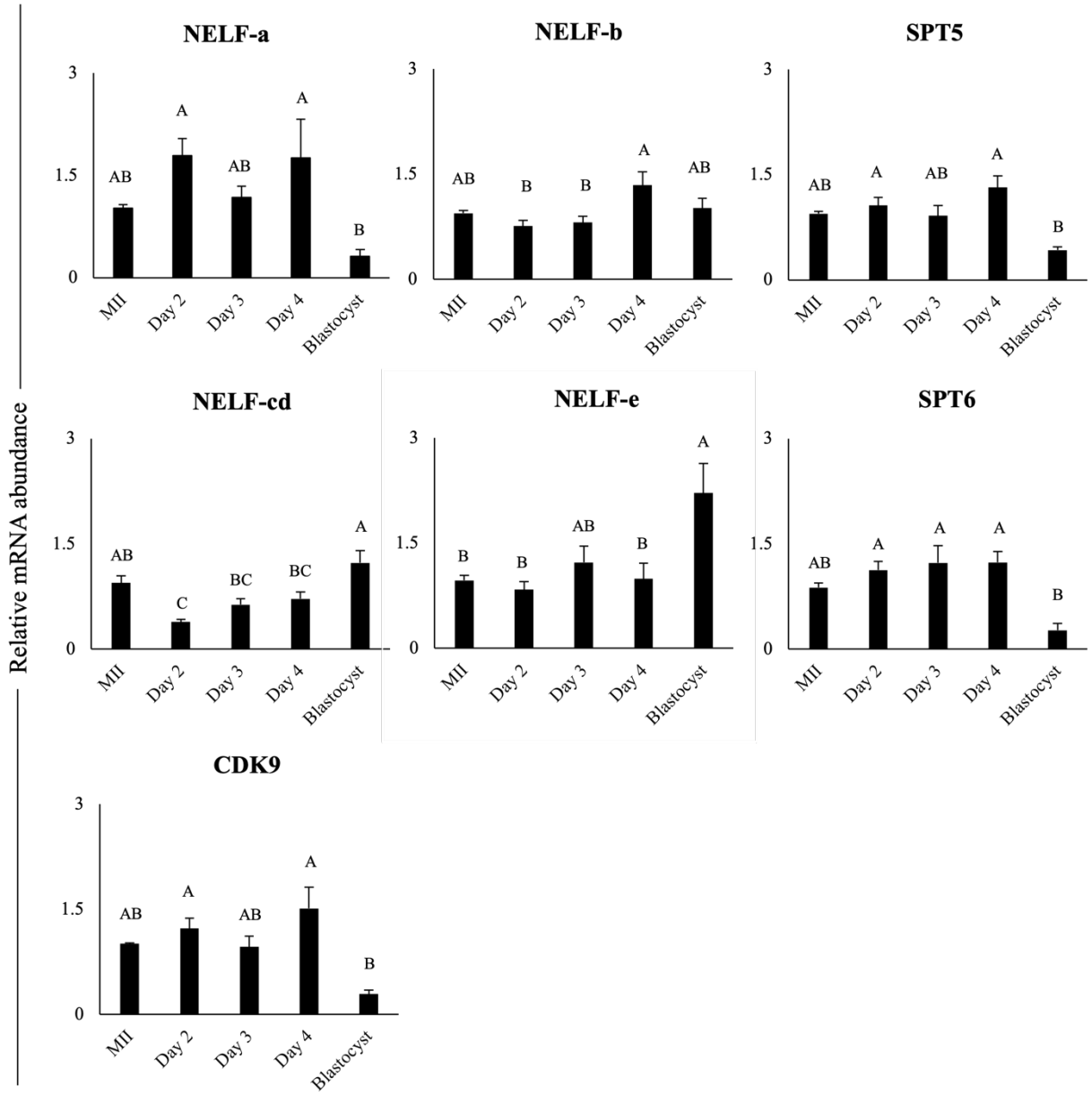

Figure 1. Relative mRNA abundance of the NELF complex and related genes in porcine oocytes and embryos. Different capital letters above bars indicate statistical differences ($P<0.05$) between developmental stages. Samples were collected from a total of 60 oocytes or embryos per treatment, divided into 4 replicates, each containing 15 oocytes or embryos per treatment and replicate.

Efficiency of *NELF-a* and *NELF-b* mRNA attenuation on day 2 and day 3 embryos

To confirm the effectiveness of mRNA attenuation, matured oocytes were microinjected with DsiRNAs targeting either *NELF-a* mRNA (si-NELF-a), *NELF-b* mRNA (si-NELF-b), or both mRNAs (si-NELF-ab). Relative mRNA abundance was assessed by q-PCR on day 2 (~48h post microinjection) or day 3 (~72h post microinjection) of embryo development and compared to control samples (si-CT) at the same developmental stage. The knockdown efficiency on day 2 was 86.72% for *NELF-a* and 78.36% for *NELF-b* mRNAs, while on day 3, it was 90.18% for *NELF-a* and 94.62% for *NELF-b* mRNAs (Figure 2). Similar attenuation levels for each gene were observed in embryos injected with DsiRNAs targeting only one mRNA (either si-NELF-a or si-NELF-b) or a combination of DsiRNAs targeting both mRNAs (si-NELF-ab) (Figure 2).

Moreover, the relative abundance of the mRNA not targeted by the injected DsiRNAs was not statically different from the control samples (si-CT), indicating the specificity of the attenuation (Figure 2). These results confirm that either one or both *NELF-a* and *NELF-b* mRNAs can be specifically attenuated in porcine embryos during early developmental stages, enabling the investigation of their specific roles.

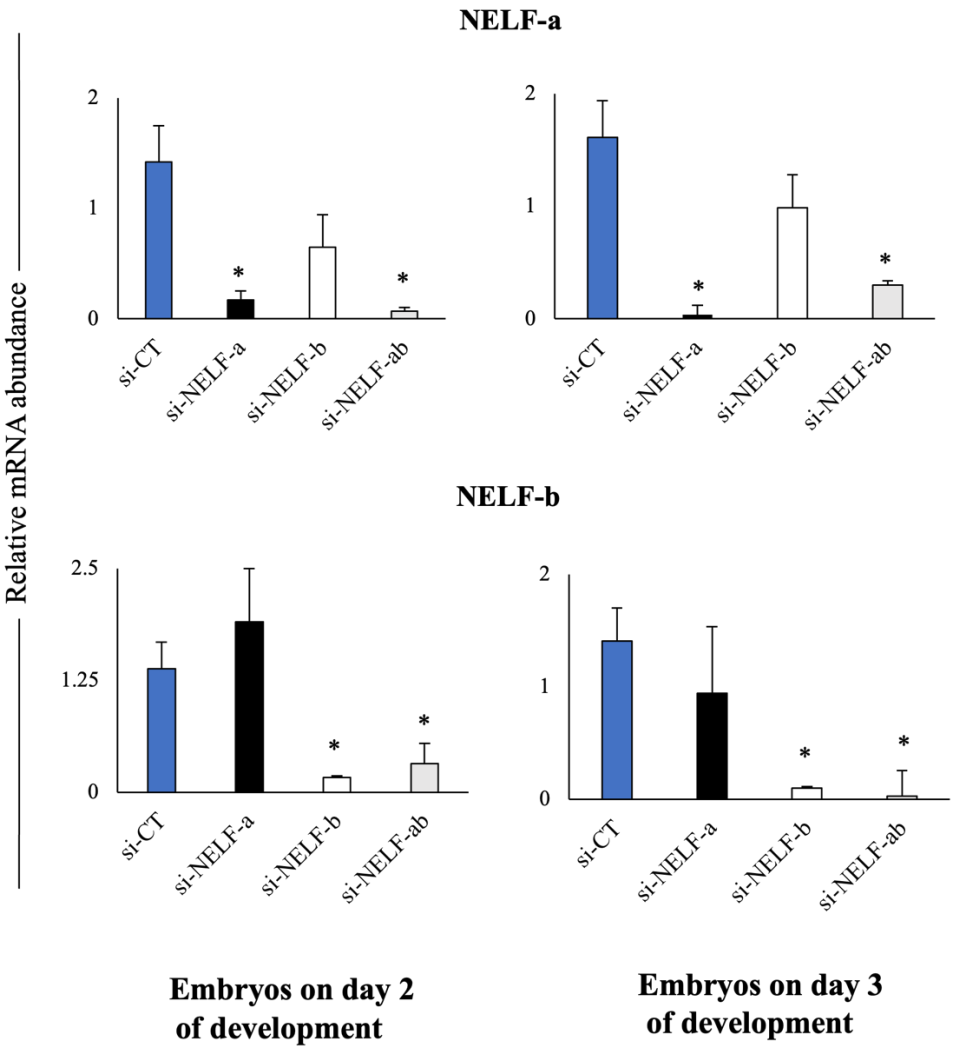

Figure 2. Attenuation of *NELF-a* or *NELF-b* mRNAs in porcine embryo. Asterisks indicate statistical differences (P<0.05) for each treatment compared to the control (si-CT) group. Samples are from three individual replicates, each containing 11 to 15 embryos per treatment and replicate.

Attenuation of NELF-a and NELF-b mRNAs decreases embryo development

Attenuation of *NELF-a*, *NELF-b* or both *NELF-a* and *NELF-b* mRNAs did not impact the initial development of embryos, as cleavage rates were similar across all treatments (Figure 3).

However, progression to the blastocyst stage was significantly reduced when attenuating either of the two components of the NELF complex individually or both, underscoring the crucial role of the NELF complex in regulating pre-blastocyst stage embryos (Figure 3). Notably, the absence of an additive impact on embryo development when both *NELF-a* and *NELF-b* mRNAs were attenuated suggests their involvement in the regulation of the same pathways.

The total number of cells in embryos that reached the blastocyst stage was not significantly decreased in the attenuated groups compared to control embryos (Figure 3), indicating that their overall quality remained largely unaffected. Interestingly, cell counting analyses of embryos that cleaved but failed to progress to the blastocyst stage revealed a superior number of nuclei in control embryos compared to embryos in all the attenuated groups (Figure 4A). Furthermore, when categorizing developmentally arrested embryos as early arrested (2-4 cells) or late arrested (5 or more cells), we observed that a higher proportion of embryos from the attenuated groups were at the early arrested stage group, while the control group had a lower proportion of early arrested embryos (Figure 4B). This observation suggests that attenuated embryos may have failed to effectively activate the embryo genome, a critical condition for the successful development of porcine embryos beyond the 4-cell stage.

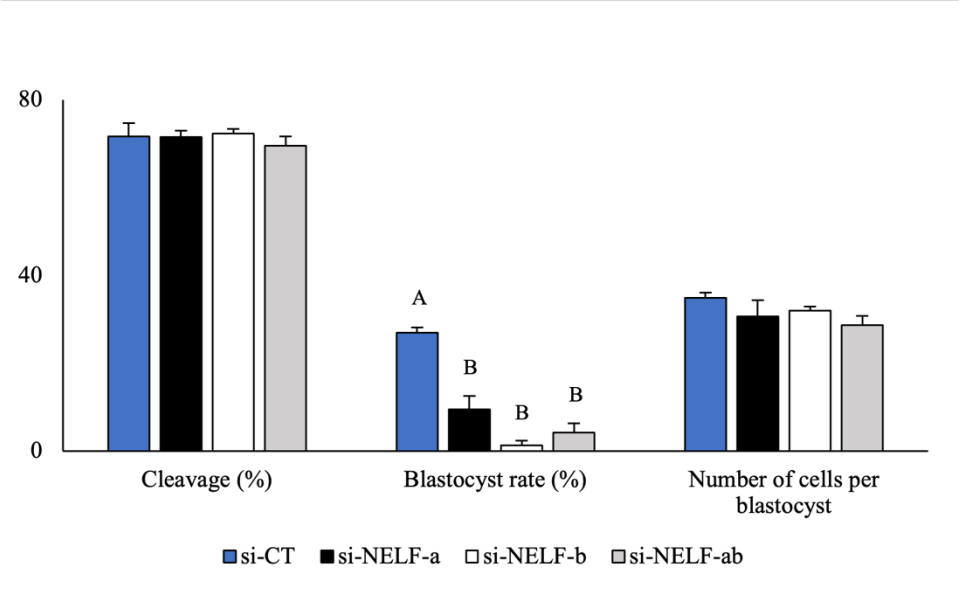

Figure 3. Effect of *NELF-a* and *NELF-b* attenuation on embryo development. Different capital letters above bars indicate statistical differences ($P<0.05$) between treatments. The experiment was repeated 4 times. The number of embryos used was 262, 243, 267, and 219 to assess cleavage, and 100, 53, 52, 72 to evaluate blastocyst rates, for the treatments si-CT, si-NELF-a, si-NELF-b, and si-NELF-ab, respectively. Total cell numbers were counted in 80, 10, 9, and 9 blastocysts from 8 replicates for the treatments si-CT, si-NELF-a, si-NELF-b, and si-NELF-ab, respectively.

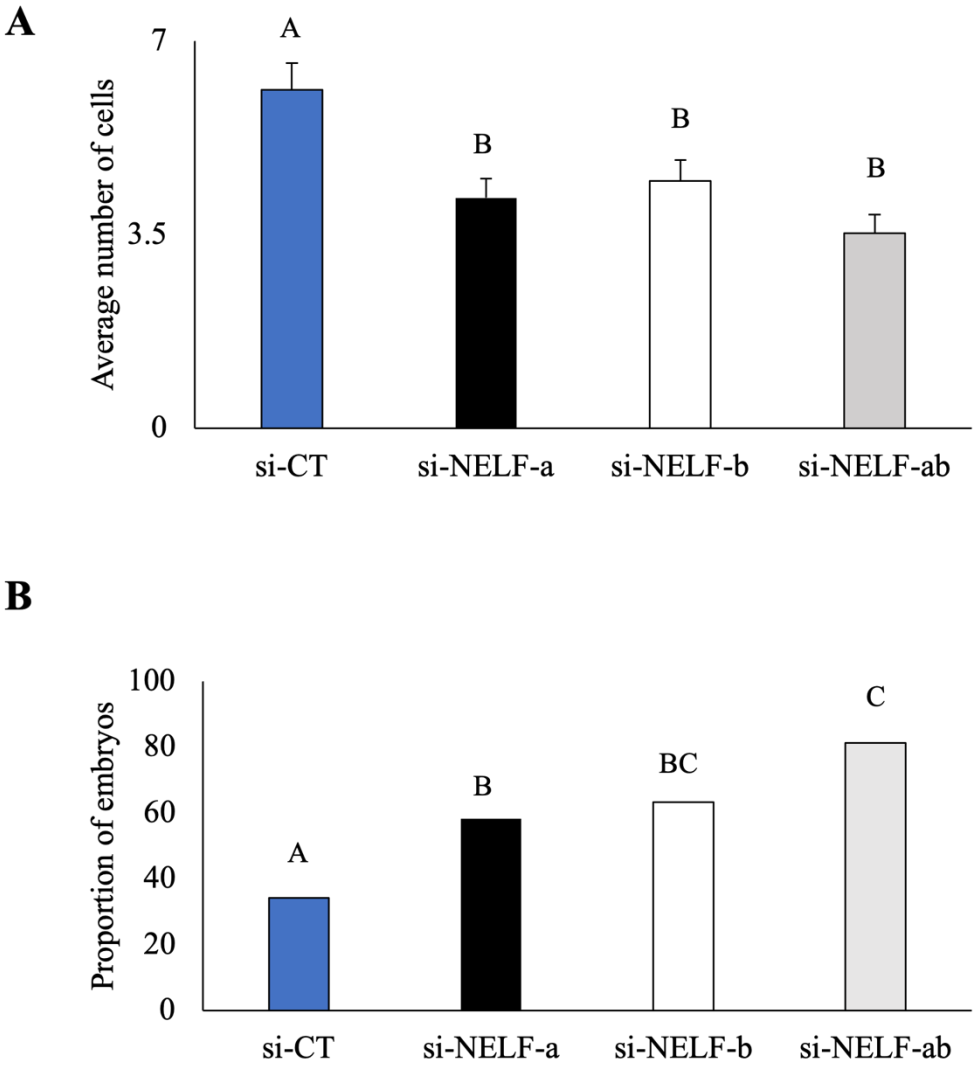

Figure 4. Cell figures from embryos that cleaved but did not reach the blastocyst stage. A) Average number of cells in control and attenuated embryos that cleaved but failed to progress to the blastocyst stage. B) Proportion of early arrested embryos at 2-4 cell stage. Different capital letters above bars indicate statistical differences ($P<0.05$) compared to control embryos (si-CT). For si-CT, si-NELF-a, si-NELF-b, or si-NELF-ab treatments, the respective number of embryos used were 41, 36, 46 and 32 respectively. Embryos used to count cell numbers were from four different replicates.

Effect of NELF-a and NELF-b attenuation in the expression of EGA related genes

To investigate whether the attenuation of *NELF-a and NELF-b* mRNAs had an impact on EGA, q-PCR analyses were performed in samples from control (si-CT) and attenuated embryos (si-NELF-a, si-NELF-b or both si-NELF-ab) to determine the relative abundance of transcripts for important EGA regulators on days 2 and 3 of development. Initially, we confirmed that the relative mRNA abundance of the selected EGA markers (*DUX*, *DPPA2*, *EIF1AX*, and *KDM5B*) increased from day 2 to day 3 of development in samples of control embryos that were injected with si-CT (Figure 5). Interestingly, comparisons between control (si-CT) and attenuated (si-NELF-a, si-NELF-b or si-NELF-ab) embryos on day 2 of development revealed a noticeable trend towards increased mRNA levels of *DUX*, *EIF1AX*, and *KDM5B* in the attenuated groups compared to the control group (Figure 6). The increase in the mRNA abundance on day 2 of development was significantly different between si-NELF-a and si-CT for *KDM5B*, and between si-NELF-ab and si-CT for *DUX* and *EIF1AX* (Figure 6). On the other hand, attenuation of either members of the NELF complex appears to prevent the normal increase in the mRNA expression of EGA regulators.

This is evident in the significantly lower abundance of *DPPA2* mRNA in si-NELF-a, si-NELF-b and si-NELF-ab embryos compared to si-CT, as well as the markedly reduced levels of *DUX* mRNA in si-NELF-a and si-NELF-ab compared to si-CT embryos (Figure 6). These findings suggest an important role of the NELF complex in the regulation of EGA in porcine embryos.

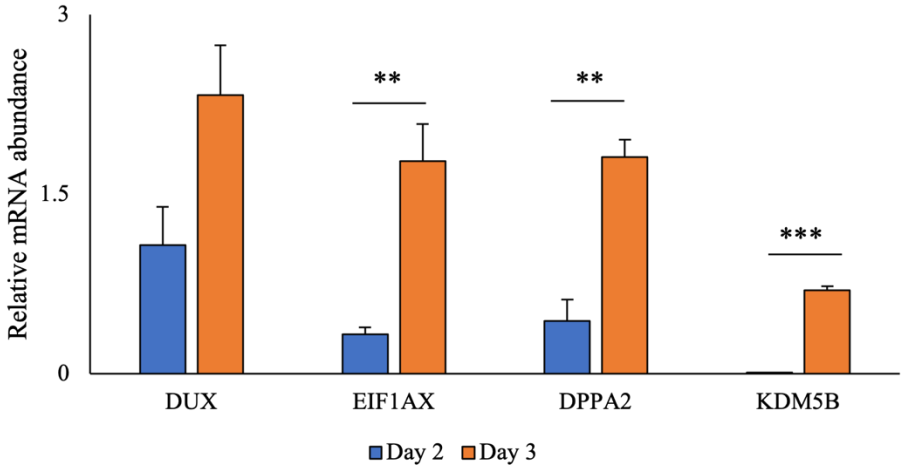

Figure 5. Relative mRNA expression of EGA markers on Day 2 and Day 3 of development of si-CT microinjected embryos. Single, double, and triple asterisks indicate statistical differences at the level of $P<0.05$, $P<0.01$ and $P<0.001$, respectively. Samples are from three individual replicates, each containing 11 to 15 embryos per treatment and replicate.

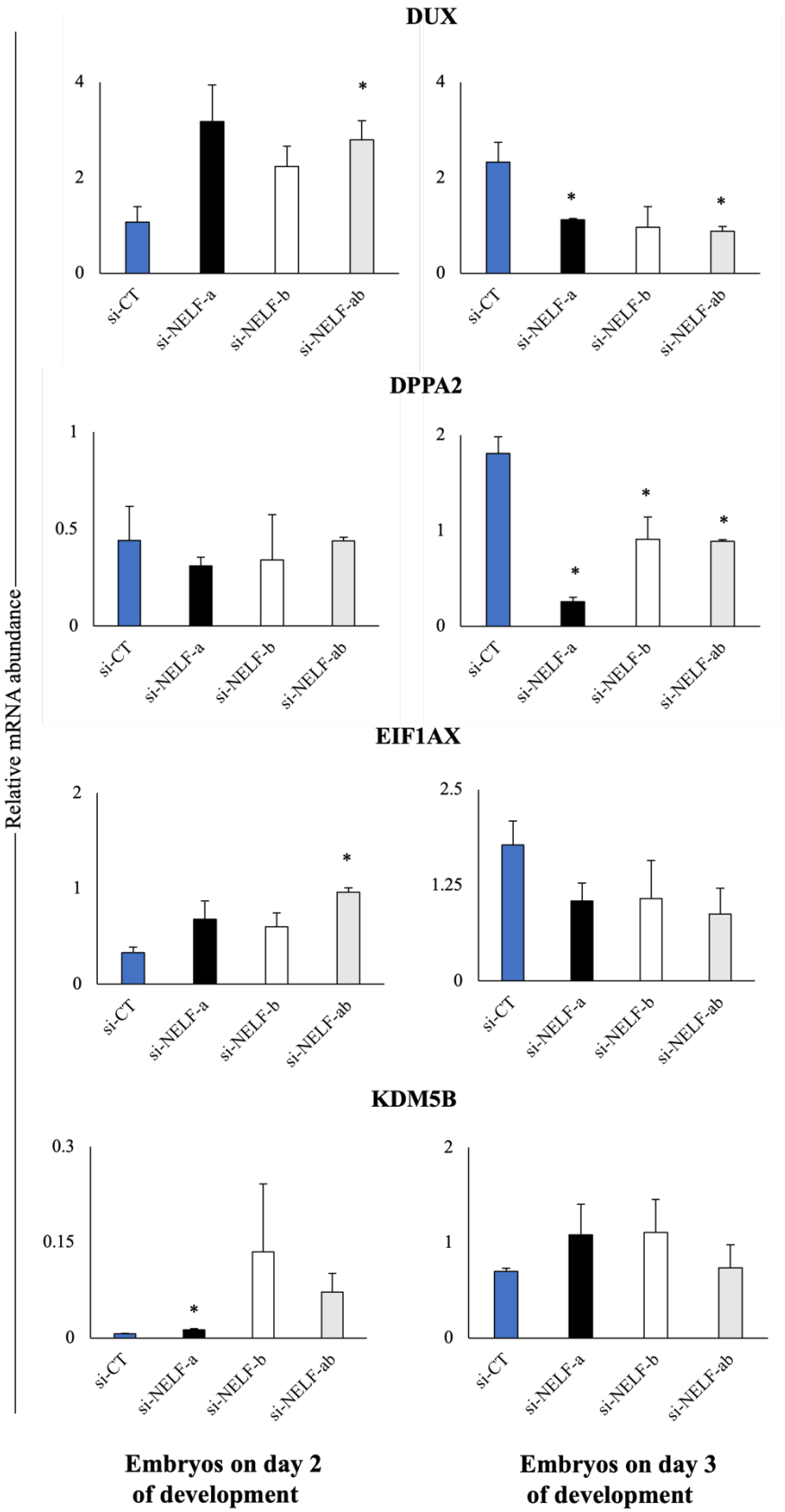
DUX
DPPA2
EIF1AX
KDM5B
Relative mRNA abundance
si-CT
si-NELF-a
si-NELF-b
si-NELF-ab
Embryos on day 2 of development
Embryos on day 3 of development

Figure 6. Relative abundance of transcripts encoding EGA related genes. Samples for q-PCR analyses were from embryos of 3 different replicates each containing 11 to 15 embryos per treatment and replicate from control (si-CT) and attenuated (si-NELF-a, si-NELF-b or si-NELF-ab), and were collected on day 2 or day 3 of development. Asterisks indicate statistical differences ($P<0.05$) compared to control (si-CT).

4.4 Discussion

Proper control of transcription during EGA is vital for normal embryonic development [15, 21, 22]. Conserved transcription regulatory mechanisms in eukaryotes include the promoter-proximal pausing, an event mainly driven by three transcription elongation factors: P-TEFb, DSIF, and NELF [23, 24, 25]. Previous research has considered the promoter-proximal pausing as a simple repressor of gene expression [14]. Alternatively, it has been suggested to be a checkpoint mechanism for fine-tune gene expression and priming genes for further or future activation [27].

The relevance of NELF's role in regulating RNA pausing has recently started to emerge [5]. However, genetic evidence for the physiological consequences of NELF-mediated stalling of polymerase pausing and its genetic regulation in early embryo development is still lacking. In this study, we explored the roles of NELF-a and NELF-b in the context of porcine embryonic development. This expands on previous findings in mice, where NELF-a was identified to participate as a key driver in the 2-cell-like cell stage, a critical reprogramming event in mESCs [4], and NELF-b was found to play an important role in early development by regulating cell differentiation in mESCs [5].

Initially, we assessed the mRNA expression of NELF complex members and other transcription-related genes during the early stages of porcine embryo development. Our findings revealed an increase in *NELF-a* and *NELF-b* mRNA levels during early embryonic developmental stages, followed by a decrease at the blastocyst stage. These results align with established functions of the NELF complex in mammalian development, as reported in previous studies [5, 28, 29], suggesting that the rise in mRNA levels coincides with the transition from a non-transcriptional to a transcriptional phase in embryonic development. Although we could not assess attenuation efficiency at the protein level due to the unavailability of tested antibodies for porcine species, our

observations suggest that NELF-a and NELF-b likely play essential roles in initiating transcription and orchestrating events associated with EGA in porcine embryos. In contrast, *NELF-cd* and *NELF-e* mRNA presented a similar pattern of expression, with an increase at the blastocyst stage, suggesting their involvement in late developmental processes, such as cell differentiation events. However, their specific roles in coordinating mammalian developmental functions have not been previously reported. The abundance of *Spt5, Spt6*, and *CDK9* mRNA displayed a fluctuating pattern at the different developmental stages, with a noticeable decrease at the blastocyst stage. This expression pattern may be associated with their key roles in RNA pausing and elongation processes [28, 30].

In our experiments, we successfully achieved knockdown efficiencies of 85% for *NELF-a* and 80% for *NELF-b* mRNAs on day 2, and 90% for *NELF-a* and 95% for *NELF-b* on day 3 following the microinjection of specific DsiRNAs. These knockdown efficiencies are consistent with those reported in other studies where DsiRNAs were microinjected to reduce the mRNA levels of various genes in porcine embryos [31]. While we followed standard microinjection procedures, it is important to acknowledge that inherent differences among embryos and minor technical variations may have contributed to not achieving 100% knockdown efficiency. In line with this, recent studies in our lab (Guay et al.; unpublished), demonstrated that, through a coinjection approach of DsiRNA tools and mRNA for the red fluorescent protein, effective intraoocyte delivery is not consistently achieved in a subset of pig oocytes via microinjection. This inconsistency likely emerges from variations in injected volume, injection positioning, and removal of the microneedle injection, as well as inherent variations in oocyte quality.

We found that the attenuation of *NELF-a, NELF-b* or both *NELF-ab* mRNAs did not disrupt embryo cleavage rates compared to control embryos injected with scrambled DsiRNA

sequences. Importantly, blastocyst formation rates were significantly impaired in all attenuated groups compared to the control group. However, the simultaneous attenuation of both *NELF-a* and *NELF-b* (NELF-ab) mRNAs did not show a synergistic impact on blastocyst development. Given the crucial roles of NELF-a and NELF-b in transcription regulation [28, 32, 33, 38], one possible explanation for this result is that the attenuation of their transcripts may lead to incorrect or incomplete transcriptional activity in the developing embryos, which in turn hinders proper development. Based on these findings, we propose that both NELF-a and NELF-b play vital roles as regulators of cellular events preceding blastocyst formation in porcine embryos.

In attempting to identify potential embryo functions accounting for the poor development observed in NELF-a, NELF-b, or NELF-ab attenuated embryos, we conducted a cell count to determine if embryos that cleaved but did not reach the blastocyst stage were blocked at a specific stage of development. On average, embryos from the attenuated groups had a 15% to 30% reduction in cell count compared to the control group. Additionally, we found that the attenuated groups tended to arrest at early stages of development (2-4 cell stage), during the EGA transition, when compared to the control embryos. This overall decline in cell numbers in the attenuated groups further suggests a disruption in the EGA transition, likely due to abnormal regulation in the expression of genes involved in EGA regulation and embryo development [34, 35]. In line with this hypothesis, previous studies conducted in mouse embryos, where early embryo development arrest was observed at the cleavage stages, indicated an essential role for NELF-b in the regulation of early embryo development [36].

Our final goal was to evaluate possible disturbances in EGA regulation. For this, we analyzed the mRNA abundance of selected EGA markers (DUX, DPPA2, EIF1AX, and KDM5B) [10] during critical days of transcription activation. DUX and DPPA2 have been identified as

inducers of EGA genes [11, 12], while KDM5B is implicated in the regulation of porcine embryo development and the DNA damage response [10], and EIF1AX is an important indicator of embryo developmental potential [37]. Upon analyzing the control injected groups on day 2 of development, we observed a consistent upward trend in the relative expression levels of these EGA markers, corresponding with the initiation of transcription from the embryonic genome, corroborating their utility as EGA references. Significantly, we observed that *DUX* and *EIF1AX* mRNA levels were significatively higher on day 2 of development in si-NELF-a and si-NELF-ab embryos, suggesting an unproper initiation of embryonic transcription [15, 34, 35]. In addition, on day 3 of development, attenuated embryos displayed an overall trend of decreased mRNA levels of the *DUX, DPPA2* and *EIF1AX* genes when compared to control embryos. Notably, significantly lower transcript levels of *DPPA2* mRNA were displayed in the attenuated groups, and lower levels of *DUX* mRNA were detected in *NELF-a* and *NELF-ab* attenuated embryos compared to the control group. These findings provide further evidence that the attenuation of NELF-a and NELF-b dysregulated the transcriptional activity of porcine embryos during the EGA transition.

In summary, the findings from this study provide compelling insights into the significance of *NELF-a* and *NELF-b* in early porcine embryonic development. The attenuation of *NELF-a* and *NELF-b* significantly impaired embryo development up to the blastocyst stage, which appears to be a consequence of the altered mRNA expression of essential genes in EGA regulation.

Author contributions

M.E.C.H., W.G.G., and V.B. conceptualized the experiments. M.E.C.H. was responsible for conducting the experiments, data analysis and writing the manuscript. W.G.G., M.P.M., L.C., K.G., Z.G., H.B. and V.G. assisted with embryo production experiments, data collection and

analysis. M.E.C.H. and V.B. performed data analysis and edited the manuscript. All authors contributed to the manuscript final review and editing.

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Data Availability Statement

The datasets generated for this study are available on request to the corresponding author.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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CHAPTER 5: DISCUSSION

Embryonic genome activation relies in the orchestration of several checkpoints and biological timed events, such as the availability, content, or activity of transcription factors. Effective transcription processes are vital for normal development and regulation of cellular functions in living organisms, including cell reprogramming and cell differentiation [172, 181-184]. In this study, our aim was to provide additional insights into the regulation of EGA by investigating the roles of NELF-a and NELF-b, which are both known to be crucial subunits of the NELF complex [185, 186]. Previous research has identified NELF-a participation in biological events, such as the regulation of the 2CLC stage, which indicates that mouse cells can be reprogrammed to a totipotent stage [16, 187], while NELF-b has been identified as a contributor to the maintenance of the undifferentiated state of mESCs [17, 79, 185, 188]. These findings underscore the relevance of NELF-a and NELF-b and highlight their potential involvement in the coordination of early embryonic development.

The first objective of our research was to investigate the expression of members of the NELF complex and other transcription-related genes during early developmental stages in porcine embryos. Our findings revealed that *NELF-a* and *NELF-b* mRNA levels increased during the early stages of development, followed by a decrease in the blastocyst stage. This observation aligns with expectations, as the increase in mRNA levels coincides with the transition from a non-transcriptional to a transcriptional state during embryonic development. Given the known roles of the NELF complex in transcription regulation in mammals [17, 178], our findings suggest that NELF-a and NELF-b may play critical roles in initiating transcription and coordinating EGA in porcine embryos. On the other hand, we observed that the remaining subunits of the NELF complex, *NELF-cd* and *NELF-e*, displayed a similar pattern of expression, which was marked by

a significant increase in their mRNA abundance at the blastocyst stage. The observed mRNA expression pattern suggests that these subunits may be involved in cell differentiation and/or the formation of the blastocele. However, it is important to note that their specific roles in regulating these functions have not been documented in the existing literature, highlighting the necessity for further investigation.

We identified that the mRNA levels of NELF complex-related genes *SPT5, SPT6,* and *CDK9* exhibit fluctuations at different stages of embryo development, with a noticeable decrease at the blastocyst stage. The relatively constant pattern in mRNA levels of these genes may be linked to their critical roles in RNA pausing and elongation processes [189], making them required at the different stages of development. In support of this hypothesis, previous studies have shown that the chemical inhibition of *CDK9* activity impaired development of mouse and pig embryos, preventing them from reaching the blastocyst stage [182, 190]. Moreover, both *SPT5* and *CDK9* seem to play major regulatory roles for normal EGA in mouse embryos [178]. Nonetheless, further research is required to fully characterize the specific roles of *SPT5, SPT6* and *CDK9* at the various stages of early porcine embryo development. After characterizing their mRNA expression profiles during early embryo development, our next objective was to evaluate whether *NELF-a* and *NELF-b* transcripts are required for normal development of porcine embryos. First, the attenuation efficiency of *NELF-a* and *NELF-b* mRNAs, by means of microinjection of DsiRNAs, was 85% and 80% on day 2 of development, and 90% and 95% on day 3 of development, respectively. The fact that mRNA analyses were performed using pools of embryos (11-15 per sample) may have accounted for the knockdown efficiency not reaching 100%. This variability could be attributed to differences between embryos in the proper delivery of the knockdown tools via microinjection. In fact, recent studies conducted in our laboratory (Guay et al. unpublished) revealed, by using a co-

injection approach of iRNA tools and mRNA for the red fluorescent protein, that proper intraoocyte delivery is not achieved in a small proportion of pig oocytes through microinjection. This issue may be attributed to various factors, including small variations in the injected volume, the positioning and removal of the injection needle, as well as inherent variations in oocyte quality.

Nonetheless, with knockdown efficiency exceeding 90% in the pool of embryos, we are confident that we have achieved a near-complete knockdown in most of the injected oocytes. In addition, our results revealed a similar knockdown efficiency when DsiRNAs targeting *NELF-a* and *NELF-b* were combined and injected together, as compared to attenuating each factor individually, which enabled exploring potential additive effects between these factors. Based on the effective results of mRNA attenuation, embryo developmental rates and embryo quality were compared between control and attenuated embryos. Cleavage rates were not perturbed by either individual or combined attenuation. However, blastocyst rates dramatically decreased in NELF-a, NELF-b, or both NELF-a + NELF-b attenuated embryos compared to control embryos. These findings clearly indicate that both NELF-a and NELF-b are important regulators of pre-blastocyst development in porcine embryos. Given the recognized roles of the NELF complex in the context of RNA Pol II pausing and release [70, 178, 181, 191] the perturbation of embryo development is not an unexpected outcome of NELF-a and NELF-b attenuation, which may have affected transcription regulation and consequently impacted EGA and embryo cell growth and differentiation. Additionally, we observed that the impact of attenuation on blastocyst formation was not significantly increased in the co-attenuated embryos. This suggests that NELF-a and NELF-b may regulate similar functions during early embryo development. Interestingly, by counting the total number of cells in embryos that reached the blastocyst stage, our findings revealed that embryo quality was not significantly impacted in the attenuated embryos that were

able to continue developing. One possibility to explain this phenotype is that the embryos that survived and developed may have undergone incomplete or low attenuation. Another possibility is that embryos with superior developmental competence, associated with better cytoplasmic maturation involving the storage of transcripts, proteins, and other molecules during oocytes maturation [90, 192, 193], were less affected by the attenuation of NELF-a and NELF-b.

In an effort to determine the stage of development at which attenuated embryos were blocked, we conducted a cell count in embryos that cleaved but were unable to reach the blastocyst stage. The average cell numbers in attenuated and co-attenuated embryos were found to be 15 to 30% lower than in control embryos that cleaved but failed to form blastocysts. Additionally, when comparing the proportion of embryos that halted development at the 2-4 cell stage, which represents the transition stage when the embryo genome is activated in pig embryos [2, 157, 160, 194], we observed a higher proportion of attenuated and co-attenuated embryos arrested as this stage compared to control embryos. This observation suggests that the attenuation of NELF-a and NELF-b affected the regulation of EGA. It is also possible that DNA damage repair was affected in the attenuated embryos. Although this aspect was not addressed in our study, previous research established that NELF-b acts as a cofactor of BRCA1, a protein implicated in DNA damage repair and transcriptional regulation [188, 195, 196]. However, this complementary role between NELF-b and BRCA1 has not been confirmed in the porcine embryo.

To gain further insights into the potential impact of NELF-a and NELF-b attenuation on EGA regulation, we assessed the mRNA abundance of four candidate genes, DUX, EIF1AX, DPPA2, and KDM5B, which are all involved in the EGA process [197, 198]. The significance of these genes in early embryo development includes the roles of DUX and DPPA2 as inducers of

EGA genes [199, 200], KDM5B in EGA regulation and DNA damage response [197], and *EIF1AX* in the normal development of embryos [198]. Notably, the relative mRNA abundance of these genes was found to significantly increase on D3 of development in porcine embryos as they became transcriptionally competent. We have confirmed this expression pattern in our study by comparing the relative mRNA expression of these genes in controls embryos on Day 2 and Day 3 of development, which further validates the utility of these genes as markers of the EGA transition.

Importantly, our results on day 2 of development revealed an overall trend of an early increase in the transcript levels of these genes in both NELF-a and NELF-b attenuated, as well as in the co-attenuated embryos, when compared to controls. Notably, *DUX* and *EIF1AX* mRNA levels were significantly higher in the NELF-ab co-attenuated embryos, indicating their premature transcription [201]. On the other hand, we observed an overall trend of decreased mRNA levels of the *DUX, DPPA2* and *EIF1AX* genes in the attenuated groups compared to control embryos on Day 3 of development. This was particularly evident through significantly lower transcript levels of *DPPA2* in all attenuated groups and *DUX* in NELF-a and NELF-ab attenuated embryos, when compared to the control group. Consistent with our findings, previous studies using mESCs have shown that NELF-a play a role in inducing a 2CLC stage via activation of DUX through its interaction with DNA topoisomerase 2a (TOP2A) [16], and *DPPA2* is known to induce the activation of *DUX* in mESCs [202]. Moreover, there is evidence from previous studies in mice suggesting a relevant role of NELF-b in the regulation of preimplantation and post-implantation stage embryos [17,203]. Collectively, our findings provide compelling evidence that the attenuation of NELF-a and NELF-b dysregulates the transcripts of important genes involved in EGA, whether increasing on Day 2 or decreasing on Day 3. Although the impact of this dysregulation on embryo development requires further investigation, prior research has indicated

that any disruption of EGA may result in embryo development arrest and the failure of embryonic implantation [17, 158, 174, 204]. This suggests that NELF-a and NELF-b are not only required for normal development up to the blastocyst stage but may also have long-term consequences for cell differentiation and embryo implantation.

In summary, the findings from this study provide robust evidence that both NELF-a and NELF-b are required for normal porcine embryonic development. The attenuation of NELF-a and NELF-b significantly impaired embryo development up to the blastocyst stage, which appears to be a consequence of the altered pattern in the mRNA expression of genes with critical roles in EGA regulation.

CHAPTER 6. CONCLUSION

The need for improvement in the Assisted Reproductive Technologies led us to analyze the key components of its fundamental basis, which relies on the understanding of the regulatory mechanisms governing early embryonic development. This process is dependent on transcriptional regulation mechanisms, where RNA pausing, more specifically the NELF complex, plays a critical role in the fate of early embryonic development. In this study the goal was to investigate the role of NELF-a and NELF-b in early porcine embryonic development. Findings reported in this thesis demonstrated that:

1) NELF-a and NELF-b transcripts are expressed in porcine oocytes and embryos.

2) Attenuation of NELF-a, NELF-b or both NELF-ab decreased early embryonic development.

3) Attenuation of NELF-a or NELF-b impaired the normal expression of EGA regulators.

4) Collectively, these findings revealed that both NELF-a and NELF-b are required for normal development of porcine embryos.

CHAPTER 7: BIBLIOGRAPHY

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