

The roles of RNA modifying enzyme
genes in preimplantation embryo
development in mice

2025

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Abbreviation

ASO	antisense oligonucleotide
ATP	adenosine triphosphate
CDX2	caudal type homeobox 2
EOMES	comesodermin
ESCs	embryonic stem cells
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
FHM	fertilization hemes-modified
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA3	gata binding protein 3
GO	gene ontology
H2AFZ	h2a histone family member z
H3K27me3	histone H3 lysine 27 trimethylation
H3K9me3	histone H3 lysine 9 trimethylation
HES1	hes family bHLH transcription factor 1
Hpi	post-insemination
HTF	human tubal fluid
IAP RNAs	Intracisternal A particle RNAs
ICM	inner cell mass
ICM	inner cell mass
IVF	in vitro fertilization
IVM	in vitro maturation
KEGG	kyoto encyclopedia of genes and genomes
KSOM	potassium-supplemented simplex optimized medium
LATS1/2	large tumor suppressor 1/2
LINE1	long interspersed nuclear element-1

M5C N5-methylcytosine
M6A N6-methyladenosine
MAP4K4 mitogen-activated protein kinase 4
METTL3 methyltransferase 3
MII Metaphase II
MST1 mammalian sterile 20-like kinase 1
MZT maternal-to-zygotic transition
NANOG Nanog homeobox
NF2 neurofibromin 2
NOTCH1 notch receptor 1
NOTCH2 notch receptor 2
NSUN2 NOL1/NOP2/Sun domain family member 2
NSUN5 NOL1/NOP2/Sun domain family member 5
NSUN6 NOL1/NOP2/Sun domain family member 6
OCT-4 POU domain class 5 transcription factor 1
OGD/R oxygen-glucose deprivation and reoxygenation
OXPHOS oxidative phosphorylation
PBS phosphate-buffered saline
PGC-1 α peroxisome proliferative activated receptor gamma coactivator 1 alpha
PVA polyvinyl alcohol
ROS reactive oxygen species
RT-qPCR reverse transcription quantitative polymerase chain reaction
SETDB1 SET domain bifurcated 1
siRNA small interfering RNA
SOX2 sex determining region Y box 2
TAZ transcriptional coactivator with PDZ-binding motif
TdT terminal deoxynucleotidyl transferase

TE trophectoderm

TEAD4 TEA domain family member 4

TRIM28 tripartite motif-containing 28

TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling

YAP1 yes-associated protein 1

YTHDC1 YTH domain containing 1

YTHDC1 YTH domain-containing protein 1

ZGA zygotic genome activation

Reagent components

Table 1. Components of mHTF, FHM, and KSOM medium

Components	Amount		
	mHTF	KSOM	FHM
NaCl	593.8 mg/100 ml	555.2 mg/100 ml	555.2 mg/100 ml
KCl	35 mg/100 ml	18.6 mg/100 ml	18.6 mg/100 ml
CaCl ₂	57 mg/100 ml	19 mg/100 ml	19 mg/100 ml
KH ₂ PO ₄	5.4 mg/100 ml	4.8 mg/100 ml	4.8 mg/100 ml
MgSO ₄ •7H ₂ O	4.9 mg/100 ml	4.9 mg/100 ml	4.9 mg/100 ml
NaHCO ₃	210 mg/100 ml	210 mg/100 ml	33.6 mg/100 ml
Glucose	50 mg/100 ml	3.6 mg/100 ml	3.6 mg/100 ml
HEPES (Acid)	—	—	238.3 mg/100 ml
HEPES (Na salt)	—	—	260.3 mg/100 ml
L-Glutamine	—	14.6 mg/100 ml	14.6 mg/100 ml
Na-pyruvate	3.7 mg/100 ml	2.2 mg/100 ml	2.2 mg/100 ml
Na-Lactate (60% syrup)	0.34 ml/100 ml	0.1852 ml/100 ml	0.1852 ml/100 ml
Streptomycin	5 mg/100 ml	5 mg/100 ml	5 mg/100 ml
Penicillin G	6.3 mg/101 ml	6.3 mg/100 ml	6.3 mg/100 ml
EDTA•2Na (10mM Solution)	—	100 µl/100 ml	100 µl/100 ml
BSA	4 mg/ ml	1 mg/ ml	1 mg/ ml
EAA (MEM)	—	1 ml/100 ml	—
NEAA	—	500 µl/100 ml	—

Table 2. Components of Acidified Tyrode's solution

Components	Amount (mg/100ml)
CaCl ₂	15
KCl	20
MgCl ₂ ·6H ₂ O	10
Na ₂ HPO ₄	4
NaCl	800
Glucose	100
PVP (K-30)	400

Table 3. Components of 0.3% PVP in PBS (PVP-PBS)

Component	Amount
Polyvinylpyrrolidone (K=30)	0.15 g
PBS (-)	50 mL
Total	50 mL

Chapter 1. General introduction

Mammalian embryonic development is a complex and highly regulated process that begins with fertilization and culminates in the formation of a fully developed organism. In mice, early embryogenesis, particularly during the pre-implantation stage, involves a series of critical events such as ZGA, compaction, polarization, and lineage specification. Understanding these processes is essential, as they lay the foundation for subsequent development and are influenced by intricate regulatory mechanisms, including RNA modifications.

1.1. The preimplantation embryo development in mice

After fertilization, the mouse embryo undergoes its first cell division to form a 2-cell embryo. At this stage, the embryo predominantly relies on maternal mRNA and proteins for its biological activities (1). A crucial event during this period is ZGA, where the embryonic genome initiates extensive transcription, replacing maternal contributions. This activation is vital for normal development, triggering new gene expression that regulates the cell cycle, differentiation, and other essential processes. During the 1-cell to 2-cell stage, the epigenetic landscape experiences significant alterations. Histone modifications such as H3K9me3 show asymmetric distribution between the paternal and maternal pronuclei, with the maternal pronucleus retaining these marks while the paternal pronucleus displays weak or absent signals. This asymmetry continues into the 2-cell stage. However, as the transition to the 4-cell stage occurs, de novo methylation of H3K9 occurs in the paternal pronucleus, resulting in a loss of the initial asymmetry in H3K9me3 distribution. This change

signifies that the paternal chromatin undergoes reprogramming to establish new H3K9me3 marks, thus aligning its epigenetic status more closely with that of the maternal chromatin (2). This reprogramming ensures that chromatin is primed for ZGA. Mitochondrial activity at this stage remains low, and the embryo relies on maternal mitochondrial content (3). In addition, ROS levels are tightly controlled to prevent DNA damage during this critical transition (4).

As development proceeds, the embryo reaches the 8-cell stage, marked by a significant morphological change known as compaction (5). This process involves increased adhesion between cells mediated by the upregulation of adhesion molecules such as E-cadherin (6). Cell shapes transform from round to flattened, and intercellular spaces narrow, forming a tightly connected cell mass. Compaction initiates the establishment of cell polarity, laying the groundwork for subsequent differentiation and playing a crucial role in cell fate determination (7). Following compaction, the embryo enters the morula stage, comprising 16 to 32 cells. During this transition from the 8-cell stage to the morula stage, histone modifications such as H3K27me3 and H3K9me3 become more prominent (8). H3K27me3 contributes to pluripotency regulation and lineage-specific gene silencing, often forming bivalent domains with H3K4me3 to mark developmental genes (9). H3K9me3 ensures genomic stability by silencing transposable elements, with stage-specific deposition on long terminal repeats at the 8-cell and blastocyst stages (10). Mitochondrial activity increases during the compaction and morula stages to support the energetic demands of compaction and cellular differentiation (11). As a result, ROS levels also rise slightly, playing a signaling role in cell fate determination. Proper regulation of ROS is essential; while low levels can promote signaling pathways (12), excess ROS can cause oxidative stress, potentially compromising embryo quality (13).

The HIPPO signaling pathway plays a key role in this process. As a conserved pathway regulating cell proliferation, apoptosis, and stem cell maintenance, HIPPO pathway activity is influenced by cell polarity and position within the embryo (14). In polarized outer cells, the pathway is inhibited, allowing YAP/TAZ transcription co-activators to enter the nucleus and promote TE-specific gene expression such as *Cdx2*. In non-polarized inner cells, the pathway is active, preventing the nuclear translocation of YAP/TAZ. The Hippo pathway also interacts with other developmental pathways, such as Notch signaling, to regulate cell differentiation and proliferation (15). The interaction between cell polarity, histone modifications, and signaling pathways ensures precise regulation of gene expression.

At the blastocyst stage, the embryonic cells differentiate into two main populations: TE and ICM. TE cells are located on the embryo's periphery. They will develop into supporting structures like the placenta. ICM cells are situated internally. They will form the embryo proper. This differentiation is regulated by multiple signaling pathways and transcription factors, including the HIPPO pathway and key factors such as *Oct4*, *Sox2*, and *Nanog* (16). The expression and localization of these factors determine cell fate, ensuring proper embryonic development. H3K27me3 and H3K9me3 are essential during this stage. H3K27me3 maintains pluripotency in the ICM, while H3K9me3 supports heterochromatin organization in both the ICM and TE, ensuring proper developmental progression (17). Mitochondrial activity peaks at the blastocyst stage (18), driven by the increased energy demands of rapid cell division and fluid accumulation in the blastocyst cavity (18-20).

For successful implantation, the blastocyst must hatch from the enclosing structure known as the zona pellucida. Hatching is facilitated by a combination of factors, including increased osmotic pressure in the blastocyst cavity and proteases

produced by TE cells (21). During the hatching stage, H3K27me3 and H3K9me3 continue to maintain gene regulation, with H3K27me3 promoting ICM cell differentiation and H3K9me3 stabilizing TE-specific gene expression (17). Mitochondrial activity supports cellular migration and invasion for implantation, While mitochondria provide ATP through OXPHOS, they also produce ROS as byproducts (22). ROS act as important signaling molecules in various reproductive processes, including embryo development and implantation (13). However, an imbalance in ROS levels can lead to oxidative stress, potentially causing cellular damage and negatively impacting reproductive outcomes (23).

1.2. Roles of RNA modifications (m5C and m6A) in cellular processes and signaling pathways

RNA modifications, particularly m5C and m6A, have emerged as critical regulators of gene expression, influencing various aspects of cell behavior. These modifications are performed by RNA methyltransferases, with Nsun5 responsible for m5C and Mettl3 for m6A. m6A is the most prevalent internal modification in eukaryotic mRNAs, significantly affecting RNA metabolism by influencing processes such as splicing, nuclear export, decay, and translation (24). These modifications can shape the stability, translation, and localization of mRNA, thereby impacting fundamental processes such as cell proliferation, differentiation, and apoptosis (25, 26). They also play a significant role in modulating signaling pathways like Hippo, influencing chromatin dynamics through histone modifications like H3K27me3 and H3K9me3, and interacting with mitochondrial function and ROS production.

The role of m5C and m6A in cell proliferation is closely tied to their ability to

regulate the stability of mRNAs that govern cell cycle progression. For instance, m6A, which is added by the METTL3 complex, can mark specific cell cycle regulators for either enhanced translation or rapid decay, depending on cellular needs (27). This dynamic regulation allows cells to quickly adapt their proliferation rates in response to external signals or stress. Meanwhile, m5C RNA methyltransferases regulate cell proliferation by modulating the expression of oncogenes (28).

In terms of differentiation, m6A serves as a molecular switch that helps embryonic and stem cells transition between states. Studies using ESCs have shown that m6A modifications are necessary for cell fate decisions, maintenance of pluripotency, and differentiation into various lineages (29). During early differentiation, m6A marks on mRNAs encoding pluripotency factors, such as Oct4 and Sox2, promote their degradation, facilitating the exit from the pluripotent state and the initiation of lineage-specific gene expression (30, 31). m6A marks are enriched in 3' untranslated regions of mRNAs encoding core pluripotency factors and facilitate the rapid degradation of unstable transcripts (31, 32). Genetic inactivation of m6A methyltransferases, such as Mettl3, impairs ESC differentiation and prolongs the expression of pluripotency genes like Nanog (31). Similarly, m5C modification helps stabilize transcripts necessary for differentiation (33), ensuring the consistent expression of key lineage-determining factors.

The balance between cell survival and programmed cell death (apoptosis) is also influenced by m5C and m6A. Proper apoptosis is crucial for development, as it eliminates unnecessary or damaged cells. Dysregulation can lead to abnormalities in tissue formation or arrest in embryo development. By stabilizing or destabilizing mRNAs encoding pro- and anti-apoptotic factors, these modifications can tilt cells toward survival or apoptosis. Recent findings suggest that RNA modifications like

m5C and m6A are involved in these processes (34). Increased m5C modifications in neurons after OGD/R may regulate stress response and apoptosis pathways (35). Research on m6A RNA modification's role in apoptosis has primarily concentrated on its mechanisms and regulatory factors, as m6A methyltransferases and demethylases can influence apoptosis by regulating related factors (36).

In early embryonic development, both m5C and m6A could play a part in the MZT, which is the stage where the control of the embryo shifts from maternal RNA to the new embryonic genome. m6A modifications have an immediate impact on mRNA fate. m6A marks facilitate both the rapid degradation and the stabilization of maternal mRNAs during MZT in mice, allowing timely activation of key developmental genes (37). This rapid adjustment in RNA stability and translation enables cells to quickly respond to developmental cues during this transition. Similarly, m5C may regulate the stability of maternal RNAs, influencing MZT (38).

The influence of RNA modifications extends into the realm of cell signaling, particularly the Hippo pathway, a key regulator of cell proliferation, differentiation, and organ size. m6A can modulate the stability of mRNAs encoding critical components of the Hippo pathway, such as *Last1* (39), thus affecting their levels in the cell. By regulating *Last1* expression, m6A indirectly shapes the outcome of Hippo signaling, impacting processes like tissue growth and cell fate determination. m5C, while less directly associated with the Hippo pathway, may influence its activity by stabilizing mRNAs of upstream regulators.

At the level of chromatin regulation, m6A interacts with histone modifications like H3K27me3 and H3K9me3, which are crucial for maintaining the transcriptional state of cells. m6A-modified non-coding RNAs, such as LINE1 and IAP RNAs, can recruit

chromatin modifiers like YTHDC1, influencing the deposition of repressive marks like H3K9me3 by recruiting SETDB1 (40). Additionally, m6A modifications can regulate the expression of chromatin-modifying enzymes such as Ezh2, thereby contributing to the regulation of H3K27me3 and affecting processes like neurogenesis and neuronal development (41).

Mitochondrial function is another critical area where RNA modifications play a role. Recent technical advances have revealed widespread but sparse occurrence of these modifications in mRNAs (42). Both m5C and m6A are present in mitochondrial transcripts, influencing mitochondrial protein synthesis and energy production. The methylation level of mitochondrial RNAs is even significantly higher than RNAs transcribed from the nuclear genome (43). m6A RNA methylation regulates mitochondrial function through promoting nuclear-encoded mitochondrial complex subunit RNA translation (44). Although NSUN2 introduces 5-methylcytosine modifications in mammalian mitochondrial tRNAs, but inactivation of NSUN2 does not significantly affect mitochondrial function (45).

1.3. Research purpose

Despite these insights, many aspects of how Nsun5-mediated m5C and Mettl3-mediated m6A modifications regulate early developmental stages, particularly before implantation, remain unclear. By focusing on both Nsun5 and Mettl3, this investigation looks into how m5C and m6A modifications have effects on important processes like proliferation, differentiation, and even apoptosis in cells. Getting knowledge about the regulatory networks that these modifications affect can advance developmental biology and might also help in reproductive health by providing

insights into better ways to use assisted reproductive technologies or solve developmental issues.

The study used various techniques to investigate the roles of Nsun5 and Mettl3 in early mouse embryonic development. Specific substances were used to target Nsun5 and Mettl3 for analysis. Immunostaining was used to observe protein expression and localization in developing embryos, while RT-qPCR and RNA-sequencing measured gene expression changes. Additionally, TUNEL Assay were conducted to evaluate apoptosis, mitochondrial function also detected by JC-1 staining.

The dissertation had two main research chapters. Chapter 2 focused on the role of the m5C methyltransferase Nsun5 in early mouse embryos, including its expression patterns, functional impact, and underlying regulatory mechanisms. Chapter 3 examined the function of the m6A methyltransferase Mettl3, particularly its role in histone modification, cell metabolism and the effect on embryonic development. The findings from these chapters were integrated to discuss the broader implications of RNA modifications in early embryogenesis, followed by conclusions and suggestions for future research.

In summary, the dissertation aimed to analyze the roles of Nsun5 and Mettl3 in early mouse embryonic development, emphasizing the importance of m5C and m6A modifications in regulating key developmental processes. By understanding how these RNA modifications impact gene expression and cellular activity during this vital phase, I can gain valuable insights into the essential processes that drive mammalian development.

Chapter 2. NSUN5 is essential for proper cell proliferation and differentiation of mouse preimplantation embryos

2.1. Introduction

The dynamic modification of RNA molecules, such as rRNA and mRNA, plays a critical role in gene expression regulation in eukaryotes (46). Specifically, m⁵C modifications are associated with the stability of rRNA and mRNA (25). NSUN5, a member of the m⁵C writer family, is known for its role in modifying rRNA and mRNA. *Nsun5* knockdown decreased clear cell renal cell carcinoma cell proliferation and increased the percentage of apoptotic cells (47). *Nsun5* knockout mice increased apoptotic cell number in brain regions (48). Previous studies have demonstrated that *Nsun5* knockout mice exhibit impaired follicular development, ovarian function, and embryonic development, including decreased blastocyst formation (38).

The Hippo signaling pathway is a crucial regulator of the segregation of ICM and TE lineages during mouse embryogenesis (49, 50). Activation of the Hippo pathway is tightly controlled by molecular mechanisms, including apical cell polarity complexes and LATS1/2 (51). The downstream effector protein YAP1 plays a redundant role in cellular responses (52, 53). The activated LATS1/2 phosphorylate YAP1 and inhibit its nuclear translocation and binding to TEAD4, which is required for the specific activation of TE-associated genes, such as *Cdx2* (51, 52). The activation of LATS1/2 relies on the presence of MOB kinase activator 1A and 1B (MOB1A/B) (54). The pivotal role of the Hippo signaling pathway in embryonic development is evident from studies demonstrating that deletion of *Mob1a/b* in mice

results in early embryonic lethality during the preimplantation stage (55).

Furthermore, the Notch signaling pathway has been identified as a key regulator of embryonic development, with its blockade leading to the downregulation of *Sox2* transcription and delayed embryo hatching (56). Recent findings have highlighted an interplay between the Notch and Hippo signaling pathways (57). YAP1/TEAD transcription factors directly regulate genes in both pathways, including trophoblast genes (*Cdx2*, *Eomes*, and *Gata3*) (53, 58, 59) and *Ccnd1* (60).

It was shown that the deposition of m6A on *Yap1* mRNA can regulate the expression of the YAP1 protein by affecting translation efficiency (61). A previous study has shown that m5C modifications in the 3' untranslated region of *Yap1* increased the stability of *Yap1* mRNA (62). NSUN6, a member of the same family as NSUN5, activates YAP target genes by participating in an RNA-protein complex that methylates MST1, a key component of the Hippo signaling pathway (63). Given these intriguing findings, I hypothesized that *Nsun5* may play a regulatory role in cell differentiation in mouse early embryos by modulating the expression of key components of the Hippo signaling pathway. To test our hypothesis, I employed an experimental approach involving microinjection of siRNA targeting *Nsun5* into fertilized zygotes. By analyzing the resulting embryos at the morula and blastocyst stages and using specific antibodies for immunostaining (YAP1 for morulae; CDX2 and OCT4 for blastocysts), I aimed to determine the impact of *Nsun5* knockdown on the cell differentiation of mouse preimplantation embryos.

2.2. Materials and methods

Re-analysis of published RNA-sequencing (RNA-seq) data

To investigate *Nsun5* expression levels during mouse preimplantation development, I analyzed the publicly available RNA-seq data from a recent study (GSE71434) (64). The fastq files (2 replicates) for MII oocytes; 1-cell, early 2-cell, late 2-cell, 4-cell, and 8-cell stage embryos; and ICM of blastocysts were quality-controlled and mapped to the mouse genome (mm10) using STAR (v. 2.7.10a) (65). The mapped reads were fed to RSEM (v. 1.3.3) (66) to calculate the transcripts per million values for *Nsun5*.

Collection of oocytes, IVF, and embryo culture

ICR female mice (8–12 weeks old; Japan SLC, Shizuoka, Japan) were superovulated by intraperitoneal injection of 7.5 IU equine chorionic gonadotropin (ASKA Animal Health, Tokyo, Japan) followed 46–48 h later by 7.5 IU human chorionic gonadotropin (ASKA Animal Health). Cumulus-oocyte complexes were collected from the ampullae of the excised oviducts at 14 h after human chorionic gonadotropin injection. Cumulus-oocyte complexes were placed in a 100- μ L droplet of human tubal fluid (HTF) medium supplemented with 4 mg/mL bovine serum albumin (BSA; A3311; Sigma Aldrich, St. Louis, MO) (67). Spermatozoa were collected from the cauda epididymis of 12–18-week-old ICR male mice (Japan SLC) and cultured for at least 1 h in a 100- μ L droplet of HTF medium. After preincubation, the sperm suspension was added to fertilization droplets at a final concentration of 1.0×10^6 cells/mL. At 2 hpi, morphologically normal fertilized embryos were collected and cultured in potassium simplex optimized medium supplemented with amino acids (68) and 1 mg/mL BSA under paraffin oil (Nacalai Tesque, Kyoto, Japan). All incubations were performed at 37°C under 5% CO₂ in air. The embryos were collected at each developmental stage after fertilization: one-cell (12 hpi), early

two-cell (24 hpi), late two-cell (36 hpi), four-cell (48 hpi), eight-cell (54 hpi), and blastocyst (96 hpi). MII oocytes were collected from cumulus oocyte complexes followed by treatment with 1% hyaluronidase to remove cumulus cells. All MII oocytes and embryos were washed three times in PBS and collected in pools of 10 to 15 for RNA extraction.

Microinjection of siRNA

Zygotes (one-cell embryos) were placed in FHM droplets during the microinjection process. siRNA molecules were microinjected into the cytoplasm of zygotes under an inverted microscope (IX73; Olympus, Tokyo, Japan) equipped with a piezo injector (PMAS-CT150; PRIME TECH, Tokyo, Japan) and a micromanipulator (IM-11-2; Narishige, Tokyo, Japan). All fertilized zygotes were microinjected with either non-targeting (control) or *Nsun5*-targeting siRNA (knockdown) within 3–6 hpi. Approximately, 10 pL siRNA (20 μ M) solution was injected into the cytoplasm of each zygote. The siRNA sequences were as follows: si*Nsun5*-1s, CCUUUCCAGGGUCUGAACAtt; si*Nsun5*-1as, UGUUCAGACCCUGGAAAGGtt; si*Nsun5*-2s, GCAAUGGAUCCAGAACCUCtt; si*Nsun5*-2as, GAGGUUCUGGAUCCAUUGCtt. Embryos were collected at 24 (2-cell) and 55 h (8-cell) after microinjection for RNA extraction to assess knockdown efficiency.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA extraction and cDNA synthesis from embryos were performed using a SuperPrepTM II Cell Lysis & RT Kit for qPCR (TOYOBO, Osaka, Japan). Synthesized

cDNA was mixed with specific primers and KOD SYBR qPCR Mix (TOYOBO), followed by RT-qPCR amplification. The protocols for RT-qPCR and measurement of transcript levels were performed as described previously (69), and *H2afz* was used as an internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (70). The following primer sequences were used for RT-qPCR:

Gapdh, 5'-GTGTTCTACCCCCAATGTG-3' (forward) and
5'-TGTCATCATACTTGGCAGGTTTC-3' (reverse);

H2afz, 5'-GGTAAAGCGTATCACCCCTCG-3' (forward) and
5'-CTTCCCGATCAGCGATTTGTG-3' (reverse);

Nsun5, 5'-TGTATTCCAGCAACTTCCAGAACC-3' (forward) and
5'-AAGGTGCGGTCTCAGCTTCTT-3' (reverse);

Lats1, 5'-AGGCGGATGTAGGAAGACCT-3' (forward) and
5'-TCCATTGCTTGGGTGAGCTT-3' (reverse);

Lats2, 5'-AAATGAGAGCCACCCCGAAG-3' (forward) and
5'-ACATCCCGCATTACCAACT-3' (reverse);

Sav1, 5'-GGATGCTGTCCCGCAAGAAA-3' (forward) and
5'-AAGGCATGAGATTCCGCAGC-3' (reverse);

Mob1a, 5'-CAGCAGCCGCTCTTCG-3' (forward) and
5'-CCTCAGATTGCCACTTCCGA-3' (reverse);

Mob1b, 5'-CGACATGAGCTTCTTGTTTGGT-3' (forward) and
5'-TGTGGCTTCCGCATGCTTTA-3' (reverse);

Map4k4, 5'-GTGACTTCCGTGGTGGGATT-3' (forward) and
5'-TTGACCACTGAGCCTTTCCG-3' (reverse);

Nf2, 5'-TGCTGTCCAGGCCAAGTATG-3' (forward) and
5'-CCTCCCACATTTCCGGAGTC-3' (reverse);

Notch2, 5'-CCGTGGGGCTGAAAAATCTC-3' (forward) and
5'-GGGTCATCTTCCGACAGCAA-3' (reverse);

Notch1, 5'-CACCAGGGTGGTCAGGAAAA-3' (forward) and
5'-GGGCAGCGACAGATGTATGA-3' (reverse);

Hes1, 5'-GAGGCTGCCAAGGTTTTTGG-3' (forward) and
5'-ACTTTACGGGTAGCAGTGGC-3' (reverse);

Sox2, 5'-AAACCACCAATCCCATCCA-3' (forward) and
5'-CCCCAAAAGAAGTCCCAAG-3' (reverse);

Cdx2, 5'-AGCTGCTGTAGGCGGAATGTATG-3' (forward) and
5'-TCAGTGACTCGAACAGCAGCAA-3' (reverse);

Gata3, 5'-CCAAGCGAAGGCTGTCTGG-3' (forward) and
5'-GTCCCCATTAGCGTTCCTCC-3' (reverse).

Detection of apoptosis by TUNEL

The blastocysts were fixed in 10% (v/v) formalin neutral buffer solution (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) for 20 minutes at 28°C and permeabilized with PBS containing 0.5% (v/v) Triton-X-100 for 20 minutes at 28°C. After washing with PBS containing 0.1 mg/ml of PVA, the blastocysts were incubated for 1 h at 37°C in 50 µl droplets of TdT -mediated TUNEL reaction mixture (In Situ Cell Death Detection Kit; Roche, Penzberg, Germany) under paraffin oil in darkness. Negative controls contained no TdT. A positive control was included by treating samples with

100 U/ml of DNaseI for 1 h at 37°C. After the TUNEL reaction, the nuclei were stained with Hoechst 33342 (Sigma-Aldrich) for 5 min. The blastocysts were washed in PBS with 0.1 mg/ml of PVA and mounted on slides in Vectashield mounting medium (Vector Laboratories, Newark, CA) and signals were observed using a fluorescence microscope (BX53 or IX73; Olympus, Tokyo, Japan). The wide-band filters for blue and UV excitation were used for the detection of TUNEL and Hoechst staining, respectively. Cells were regarded as apoptotic when they showed both TUNEL Labeling and apoptotic morphology (71). Total number of cells and number of apoptotic dead cells were counted, and dead cell indices were calculated by dividing the number of dead cells by the total number of cells, which included dead cells. Labeling and analysis were carried out blind on individual experimental groups of embryos.

Immunofluorescence analysis

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 20 min after removal of the zona pellucida with acid Tyrode's solution (pH 2.5). After washing three times in PBS containing 0.3% polyvinylpyrrolidone (K-30; Nacalai Tesque), fixed embryos were treated with 0.5% Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 40 min, and were blocked in PBST containing 1.5% BSA, 0.2% sodium azide, and 0.02% Tween 20 (antibody dilution buffer) at room temperature for 1 h. Embryos were incubated with one of the following primary antibodies in antibody dilution buffer at 4°C overnight: anti-YAP1 (1:2,000 dilution; DBH1X; Cell Signaling, Danvers, MA), anti-CDX2 (1:100 dilution; MU392A-UC; Biogenex, Fremont, CA), and anti-OCT4 (1:200

dilution; ab19857; Abcam, Cambridge, UK). Embryos were washed three times in antibody dilution buffer and then incubated with an appropriate secondary antibody in antibody dilution buffer (1:500 dilution; Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 594-conjugated goat anti-mouse IgG; Invitrogen) at room temperature for 1 h. After staining with Hoechst 33342 (Sigma-Aldrich), embryos were mounted on slides in Vectashield mounting medium (Vector Laboratories, Newark, CA) and signals were observed using a fluorescence microscope (BX53 or IX73; Olympus, Tokyo, Japan). The number of analyzed embryos is given in each figure legend. The intensities of YAP1 immunofluorescence in embryos were quantified using ImageJ software as follows. The embryos' region in the image was first delineated and the average fluorescence intensity within this region were measured and the values were termed 'mean intensity'. Concurrently, the region's areas were measured and multiplied by the mean intensity to calculate 'total intensity'.

2.3. Statistical analysis

Developmental rates were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons. Data for RT-qPCR, blastocyst diameter, cell number, and immunofluorescence intensity were analyzed using Student's *t*-test for pairwise comparisons or one-way ANOVA followed by the Tukey-Kramer test for multiple comparisons. P-values < 0.05 were considered statistically significant; 0.05 < P < 0.1 were considered a tendency toward significance.

Ethical approval for the use of animals

All experimental procedures were approved by the Animal Research Committee of Kyoto University (permit no. R3-17) and performed in accordance with the committee's guidelines. Mice were euthanized by cervical dislocation without the usage of anesthetic agents by trained personnel following the ARRIVE guidelines.

2.4. Results

siRNA efficiently knocked down the *Nsun5* expression

In my study, I employed qPCR and re-analysis of published RNA-seq data to investigate *Nsun5* mRNA expression dynamics during mouse pre-implantation development. The results of qPCR experiments were highly consistent with those revealed by RNA-seq data analysis when using *Gapdh* as an internal control gene. A significant increase in *Nsun5* mRNA expression levels was observed at the late 2-cell stage (Fig. 2-2a and b). However, when using *H2afz* as an internal reference gene, the qPCR results (Fig. 2-2c) exhibited some discrepancies compared to the RNA-seq data. This variation could be attributed to the low expression levels of *H2afz* mRNA from the MII stage to the early 2-cell stage (Fig. 2-2d), resulting in a higher relative expression of *Nsun5* mRNA at the MII stage. To efficiently knock down *Nsun5* and minimize off-target effects, I employed two specific siRNAs (si*Nsun5*-1 and si*Nsun5*-2) to target its mRNA. The qPCR results demonstrated that both siRNAs exhibited good efficiency in knocking down *Nsun5* levels at the 2-cell stage (Fig. 2-3a). I used at least one of si*Nsun5*-1 or si*Nsun5*-2 for the following experiments and confirmed significant knockdown efficiency of si*Nsun5*-1 at the 8-cell stage (Fig. 2-3b).

***Nsun5* is essential for the development of mouse preimplantation embryos**

I monitored embryo development from 24 to 120 hpi (Fig. 2-4). *Nsun5* knockdown significantly reduced the rate of mouse blastocyst formation (Fig. 2-4a and b). Remarkably, at 120 hpi, the blastocysts in the knockdown group exhibited a significant impairment in hatching from the zona pellucida when compared to the control group (Fig. 2-4a and c). Furthermore, at 96 hpi, the mean size of blastocysts was smaller in the knockdown group than in the control group (Fig. 2-4c and d). These results indicate the crucial role of *Nsun5* gene in regulating various aspects of early embryonic development, including blastocyst formation, hatching from the zona pellucida, and size development. In addition, when I extended the culture up to 144 hpi, most embryos that had not hatched by 120 hpi in the knockdown group died, with only a few being able to hatch (Fig. 2-5).

***Nsun5* knockdown decreases cell numbers and increases apoptosis in mouse preimplantation embryos**

To investigate the underlying mechanisms for the impairment of embryonic development, I performed Hoechst staining of morula and blastocyst stage embryos to count cell numbers. At both stages, the *Nsun5* knockdown group exhibited a significant decrease in total cell number compared to the control group (Fig. 2-6a and b). In addition, knockdown of *Nsun5* significantly increased apoptosis in embryonic cells. The knockdown group exhibited a significantly higher apoptotic cell index (18%) compared to the control group (10%) (Fig. 2-6c).

***Nsun5* knockdown inhibits YAP1 nuclear translocation and impairs cell**

differentiation

As mentioned earlier, *Nsun5* depletion led to a severe consequence in which blastocysts were unable to hatch from the zona pellucida (Fig. 2-4b). This failure is possibly attributed to impaired cell differentiation and hindered development of TE cells (72). The nuclear translocation of YAP1 during the morula stage is crucial for the formation of the TE (52). Therefore, to investigate the specific mechanisms underlying the inability of knockdown embryos to hatch from the zona pellucida, I performed YAP1 immunostaining on morula-stage embryos as well as CDX2 and OCT4 immunostaining on blastocyst-stage embryos.

I found that the nuclear translocation of YAP1 was inhibited at the morula stage in *Nsun5* knockdown embryos (Fig. 2-7a–d). Even when the total cell counts of the morula embryos were similar, the nuclear translocation of YAP1 in the knockdown group appeared to be lower than that in the control group (Fig. 2-7d). In addition, the mean and total amount of YAP1 protein in embryos were not different between the control and the knockdown groups (Fig. 2-7e, f). Our data revealed a lower number of CDX2-positive cells in the blastocyst stage of the knockdown group (Fig. 2-7a, g, h). In addition, there was no difference in the number of OCT4-positive cells between the knockdown and control groups (Fig. 2-7a, i), while the knockdown group exhibited a significantly higher proportion of OCT4-positive cells in the total blastocyst cell population than the control group (Fig. 2-7a, j). Moreover, the ratio of OCT4-positive cells to CDX2-positive cells was also significantly higher in the knockdown group than in the control group (Fig. 2-7k).

***Nsun5* knockdown alters the Hippo signaling pathway in mouse preimplantation**

embryos

To determine whether the impaired development observed in the *Nsun5* knockdown group was mediated through inhibition of the Hippo signaling pathway, I measured the relative mRNA levels of key genes involved in the Hippo signaling pathway at the morula stage, and assessed downstream genes controlled by the Hippo pathway that are associated with cell proliferation, cell differentiation, and hatching at the blastocyst stage. Our results demonstrated that *Nsun5* knockdown led to a significant increase in the relative mRNA levels of key genes in the Hippo signaling pathway, such as *Lats1* and *Lats2*, in the morula stage (Fig. 2-8a). Furthermore, the levels of *Mob1a* and *Mob1b*, which are also associated with the Hippo pathway, tended to increase in the *Nsun5* knockdown group (Fig. 2-8a). Additionally, at the blastocyst stage, *Nsun5* knockdown tended to increase the expression of *Notch1* (Fig. 2-8b), which is located downstream of the Hippo pathway and involved in hatching processes.

2.5. Discussion

Previous studies suggest that RNA modifications play important roles in mouse preimplantation embryo development. The reversible m6A modification has vital functions in mammalian oocyte maturation and pre-implantation embryonic development processes (73). N-acetyltransferase 10-mediated N4-acetylcytidine is a crucial regulator of oocyte maturation (74). m5C, one of the most prevalent modifications of RNA, is recruited by NSUN1–7 (75). By knocking down *Nsun5* mRNA levels, I observed impaired cell proliferation and differentiation and disrupted embryonic development with increased apoptotic cell death. Compared to our results,

Nsun5 knockout mice exhibited a greater reduction in blastocyst formation due to impaired ovarian development and poor oocyte quality (76). My study targeted *Nsun5* knockdown only from the zygote stage to explore its role in pre-implantation embryonic development. This may be the reason for more moderate influences of *Nsun5* depletion observed in the present study.

Although it is unclear why apoptosis was increased in *Nsun5* knockdown embryos, some findings suggest a relationship between the Hippo signaling pathway and apoptosis (77). For example, in Adriamycin-induced focal segmental glomerulosclerosis, nuclear exclusion of YAP1 in podocytes leads to their apoptosis (78). Therefore, it is possible that the changes in Hippo signaling in blastocysts I observed are related to increased apoptotic cell death.

In my study, even when the total cell counts of the morula embryos were similar, the nuclear translocation of YAP1 in the knockdown group appeared to be lower than that in the control group (Fig. 2-7d). This observation, coupled with the fact that the amount of YAP1 in the entire embryo was consistent across both groups, indicates that the reduced nuclear localization of YAP1 might play a significant role in the apoptosis observed in the knockdown embryos, rather than being a result of developmental delay. In addition, the impaired hatchability observed in the knockdown group even in the extended culture (Fig. 2-5) also strongly suggests that this phenomenon is not simply due to a decrease in cell number or embryo size, but rather to changes in differentiation and apoptotic signaling. The decreased cell number with increased apoptosis in *Nsun5* knockdown embryos suggests that NSUN5 plays a crucial role in regulating cell cycle progression, consistent with a previous study demonstrating that decreased NSUN5 expression impairs cell cycle progression (79). Our findings shed light on a potential connection between *Nsun5* depletion,

alterations in the Hippo signaling pathway, and the subsequent effects on YAP1 activity. *Nsun5* gene interference resulted in changes in the mRNA expression levels of key genes in the Hippo signaling pathway, such as *Lats1* and *Lats2*. LATS1 and LATS2 phosphorylate and inhibit nuclear translocation of the transcriptional co-activator YAP1 (80, 81). YAP1 plays a key role in cell fate determination (82) and tissue development (83), making it one of the most important downstream effectors of the Hippo signaling pathway (84). The observed reduction in CDX2-positive cells during the blastocyst stage in the knockdown group suggests potential implications of NSUN5 for the inhibition of YAP1 nuclear translocation (51). This reduction may lead to the failure of knockdown embryos to form blastocysts (85) and results in limited expansion, which is essential for hatching and efficient interaction with the uterine endometrium during implantation in placental mammals (86). This likely explains the challenges faced by knockdown embryos in hatching from the zona pellucida. Additionally, the altered proportions of OCT4-positive and CDX2-positive cells indicate a potential imbalance in the pluripotent and TE lineages in the knockdown embryos. These findings highlight the crucial role of NSUN5 in modulating YAP1 nuclear translocation, thereby influencing cell differentiation processes during preimplantation embryo development. Moreover, the observed developmental abnormalities in *Nsun5* knockdown embryos may be associated with dysregulation of the Hippo signaling pathway. The upregulation of key genes in the Hippo pathway and alterations in downstream gene expression provide insights into the molecular mechanisms underlying the developmental impairments observed in the knockdown embryos. However, more research is needed to reveal precisely how NSUN5 regulates these processes. Employing techniques such as RNA immunoprecipitation could uncover interacting partners and their role in Hippo

pathway regulation.

In conclusion, our findings highlight the crucial role of *Nsun5* in mouse preimplantation embryo development. Knockdown of *Nsun5* mRNA led to reduced cell numbers, increased apoptosis, compromised differentiation with hampered YAP1 nuclear translocation, and alterations in the Hippo signaling pathway. The significance of *Nsun5* suggests potential therapeutic avenues for reproductive medicine and fertility.

Figures

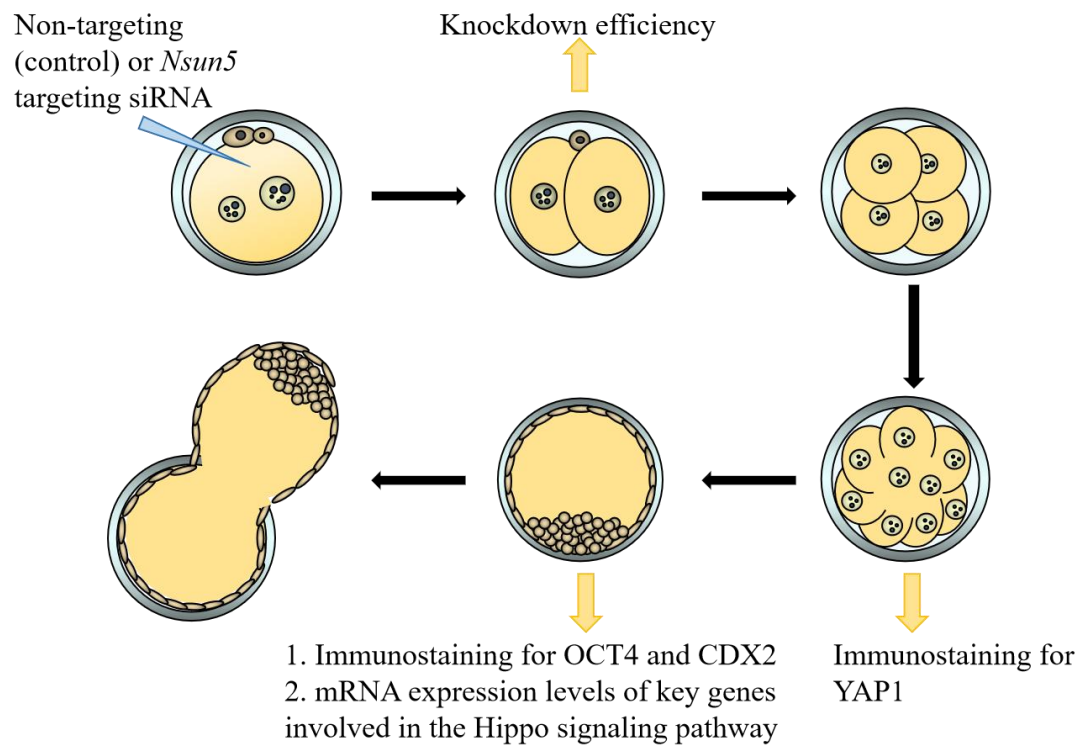


Fig. 2-1. Experimental workflow

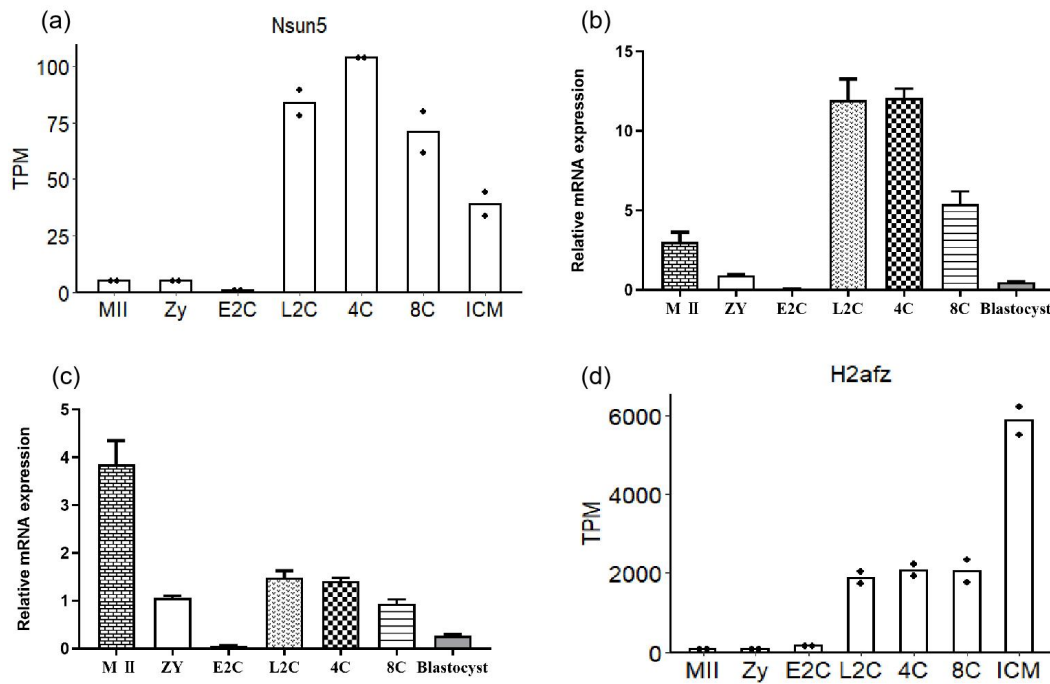


Fig. 2-2. Expression profile of *Nsun5* mRNA in mouse preimplantation embryos.

a Expression profile of *Nsun5* mRNA during the mouse preimplantation development calculated from RNA-seq data (64). **b** Dynamic changes in the relative expression of *Nsun5* mRNA during the mouse preimplantation development by RT-qPCR using *Gapdh* as an internal control gene. **c** Dynamic change in the relative expression of *Nsun5* mRNA during the mouse preimplantation development assessed by RT-qPCR using *H2afz* as an internal control gene. **d** Expression profile of *H2afz* mRNA during the mouse preimplantation development calculated from RNA-seq data (64).

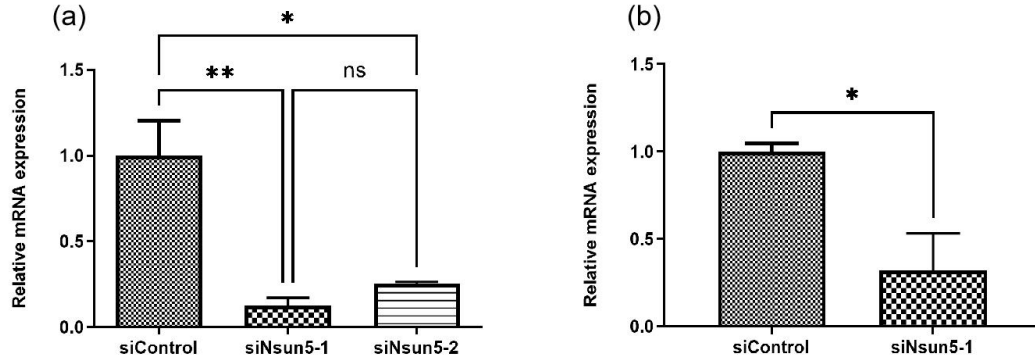


Fig. 2-3. Knockdown efficiency of siRNA treatment.

a Knockdown efficiency of siRNA treatment at the 2-cell stage. All fertilized zygotes were microinjected with siControl or two different siRNAs targeting *Nsun5* within 3–6 hpi and subjected to RT-qPCR to access knockdown efficiency at 24 h after injection. Data are expressed as means $\pm$ standard error of the mean (S.E.M.) (three replicates). Ten to 15 embryos per replicate in each experimental group were analyzed. Statistical analysis was performed using one-way ANOVA followed by the Tukey-Kramer test. Not significant (ns) $P > 0.1$, $*P < 0.05$, $**P < 0.01$. **b** Knockdown efficiency of siRNA treatment at the 8-cell stage. Ten to 15 embryos per replicate in each experimental group were analyzed. Statistical analysis was performed using unpaired t-test. $*P < 0.05$.

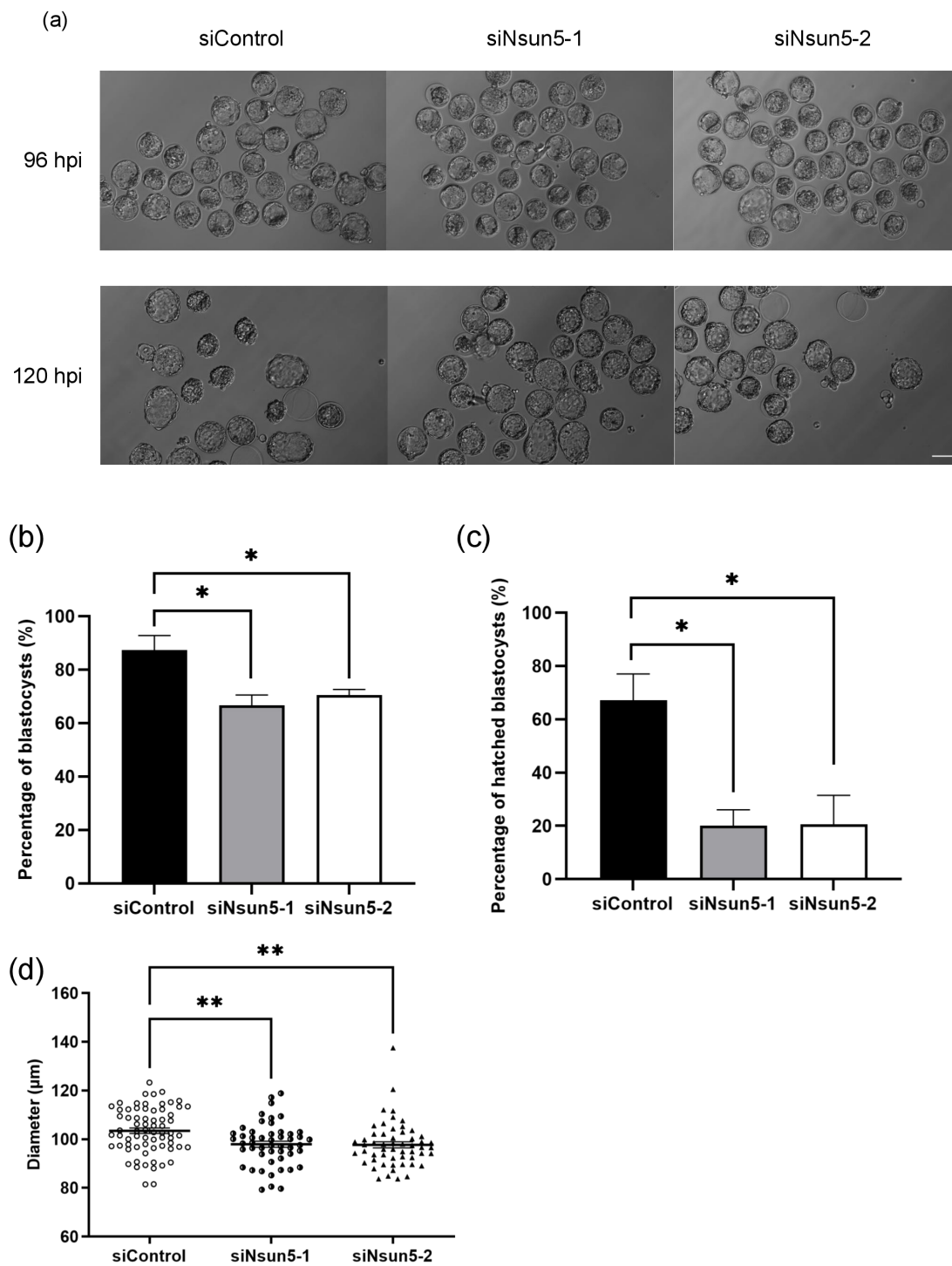
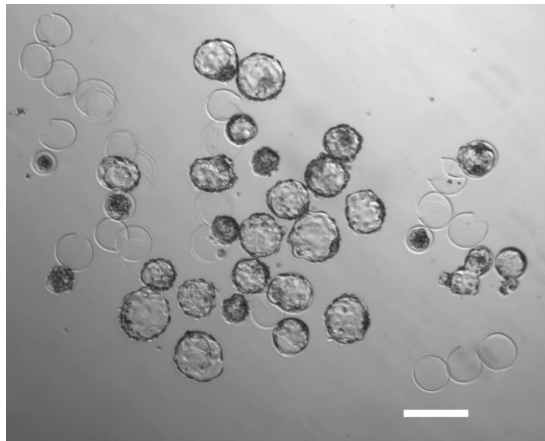


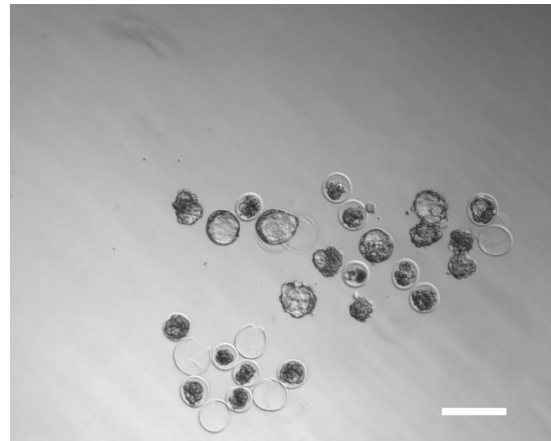
Fig. 2-4. Effects of *Nsun5* knockdown on mouse preimplantation embryo development.

a Morphology of mouse embryos after siControl or si*Nsun5* microinjection. Scale bar, 100 μm. Embryos were photographed at 96 and 120 hpi. **b** Blastocyst formation rate.

c Hatched blastocyst rate. Zygotes were cultured *in vitro* until 120 hpi and the blastocyst rate and hatched blastocyst rate were quantified. Data are expressed as means $\pm$ S.E.M. (three replicates). siControl, $n = 79$; si*Nsun5*-1, $n = 75$; si*Nsun5*-2, $n = 78$. * $P < 0.05$, one-way ANOVA followed by the Tukey-Kramer test. **d** Diameter of blastocysts. siControl, $n = 69$; si*Nsun5*-1, $n = 50$; si*Nsun5*-2, $n = 55$. ** $P < 0.01$, one-way ANOVA followed by the Tukey-Kramer test.



Control



siNsun5

Fig. 2-5. Morphology of mouse embryos after siControl or si*Nsun5* microinjection. Scale bar, 200 μ m. Embryos were photographed after the extended embryo culture at 144 hpi.

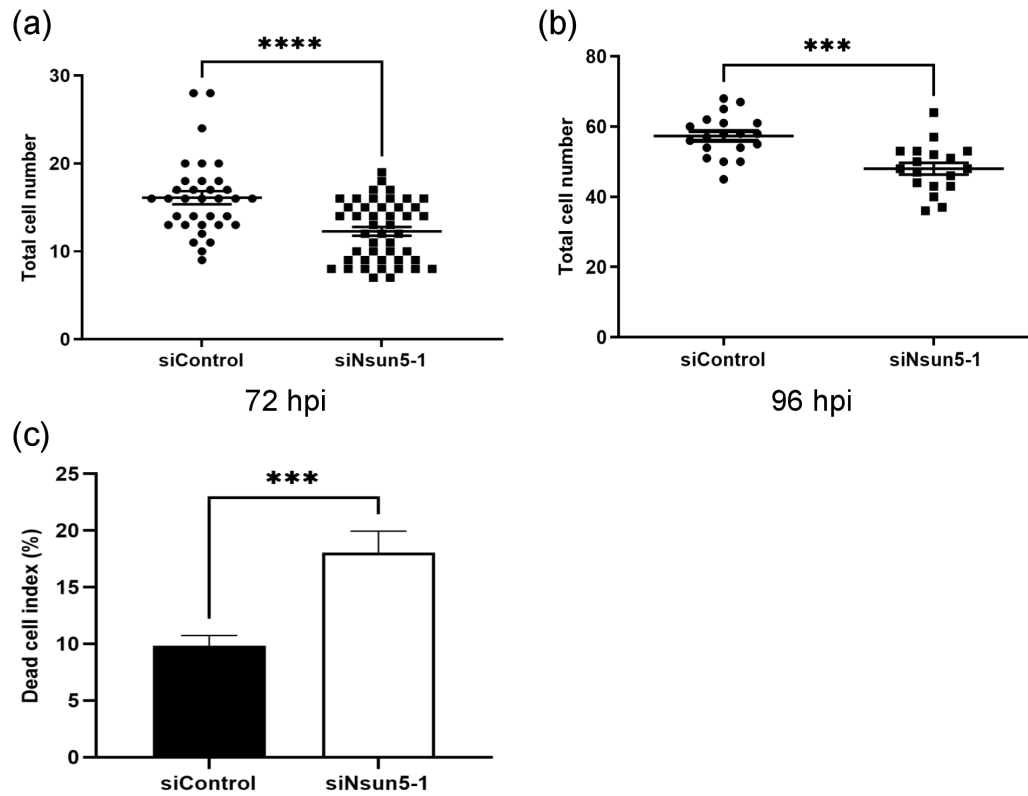
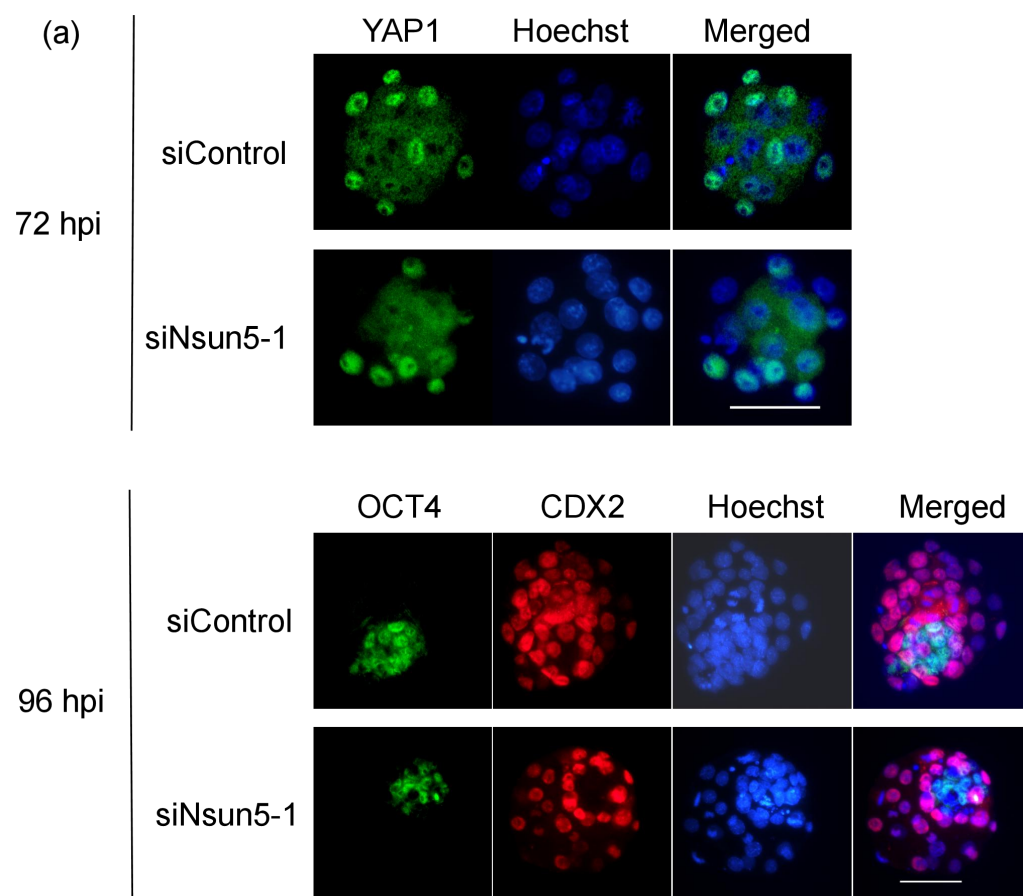
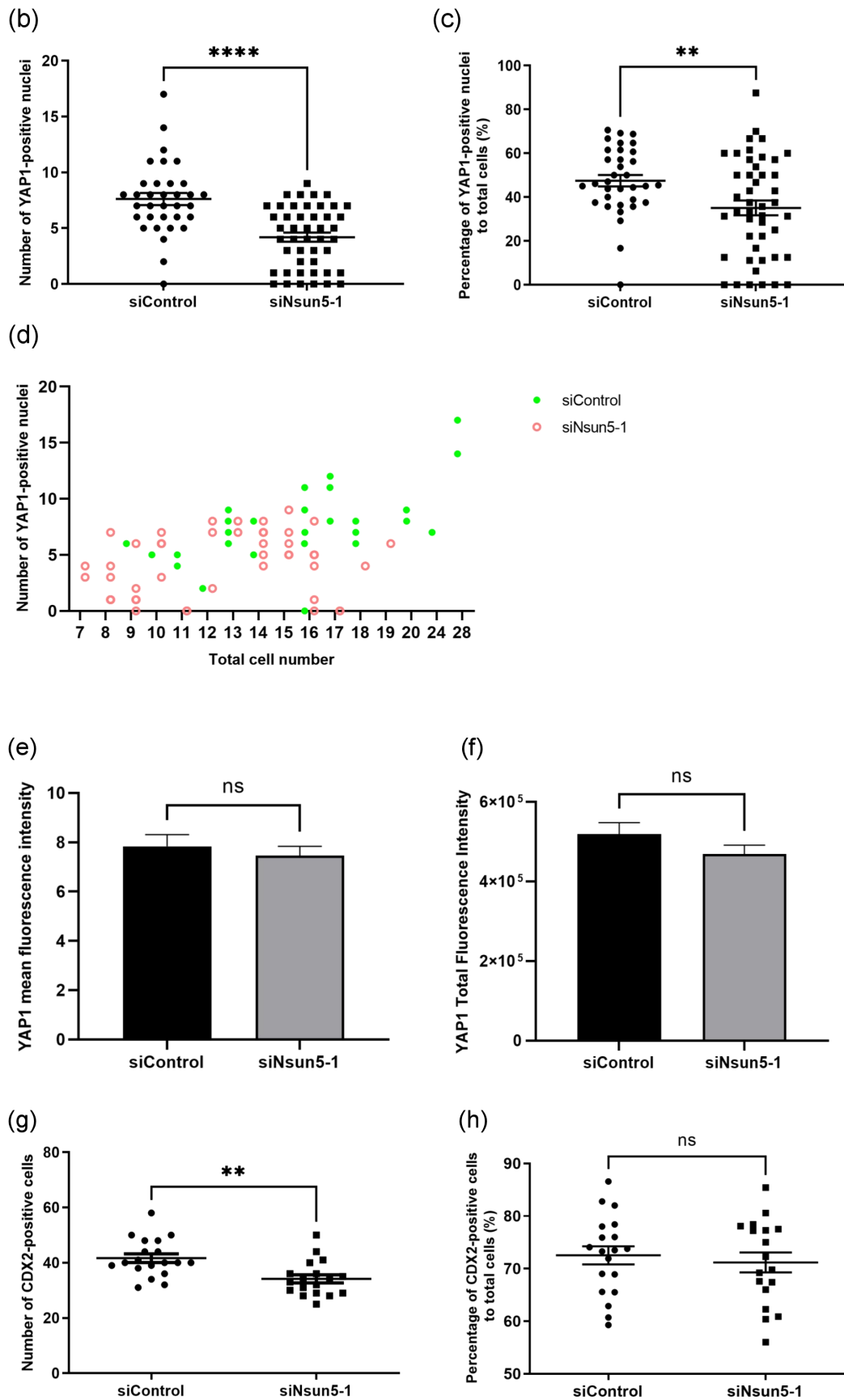


Fig. 2-6. Effects of *Nsun5* knockdown on total cell number of embryos. **a** Total cell number of mouse morula stage embryos. siControl, $n = 34$; siNsun5-1, $n = 46$. **** $P < 0.0001$, unpaired t -test. **b** Total cell number of mouse blastocyst stage embryos. siControl, $n = 19$; siNsun5-1, $n = 18$. *** $P < 0.001$, unpaired t -test. **c** Dead cell index at the blastocyst stage. siControl, $n = 42$; siNsun5-1, $n = 38$. *** $P < 0.001$, unpaired t -test.





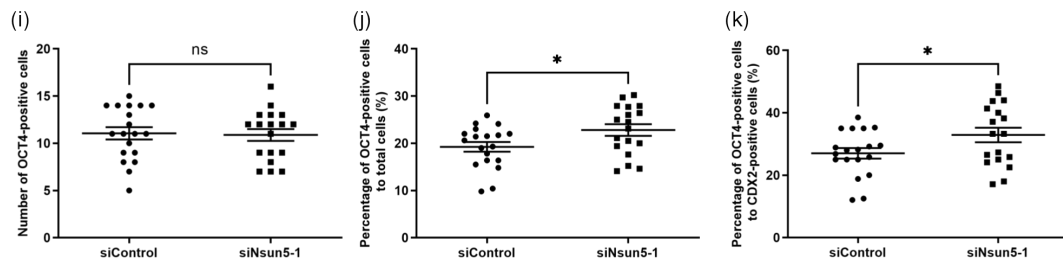


Fig. 2-7. Effects of *Nsun5* knockdown on YAP1 nuclear translocation and cell differentiation of mouse preimplantation embryos. **a** Immunostaining images of mouse preimplantation embryos at the morula (72 hpi) and blastocyst (96 hpi) stages. Scale bar, 100 μ m. **b** Number of cells that exhibited YAP1 nuclear localization at the morula stage. **c** Ratio of nuclear YAP1-positive cells to total cell number at the morula stage. siControl, $n = 34$; si*Nsun5-1*, $n = 46$. **** $P < 0.0001$, unpaired t -test. **d** Relationship between the number of total cells and the number of YAP-1 positive nuclei in morulae. **e** Mean fluorescence intensity of YAP1 throughout the embryo at the morula stage. **f** Total fluorescence intensity of YAP1 throughout the embryo at the morula stage. siControl, $n = 9$; si*Nsun5-1*, $n = 13$. Ns $P > 0.05$, unpaired t -test. **g** CDX2-positive cells at the blastocyst stage. **h** Ratio of nuclear CDX2-positive cells to total cell number at the blastocyst stage. siControl, $n = 34$; si*Nsun5-1*, $n = 46$. **i** OCT4-positive cells at the blastocyst stage. **j** Ratio of nuclear OCT4-positive cells to total cell number at the blastocyst stage. **k** Ratio of nuclear OCT4-positive cells to CDX2-positive cells at the blastocyst stage. siControl, $n = 19$; si*Nsun5-1*, $n = 18$. Ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, unpaired t -test.

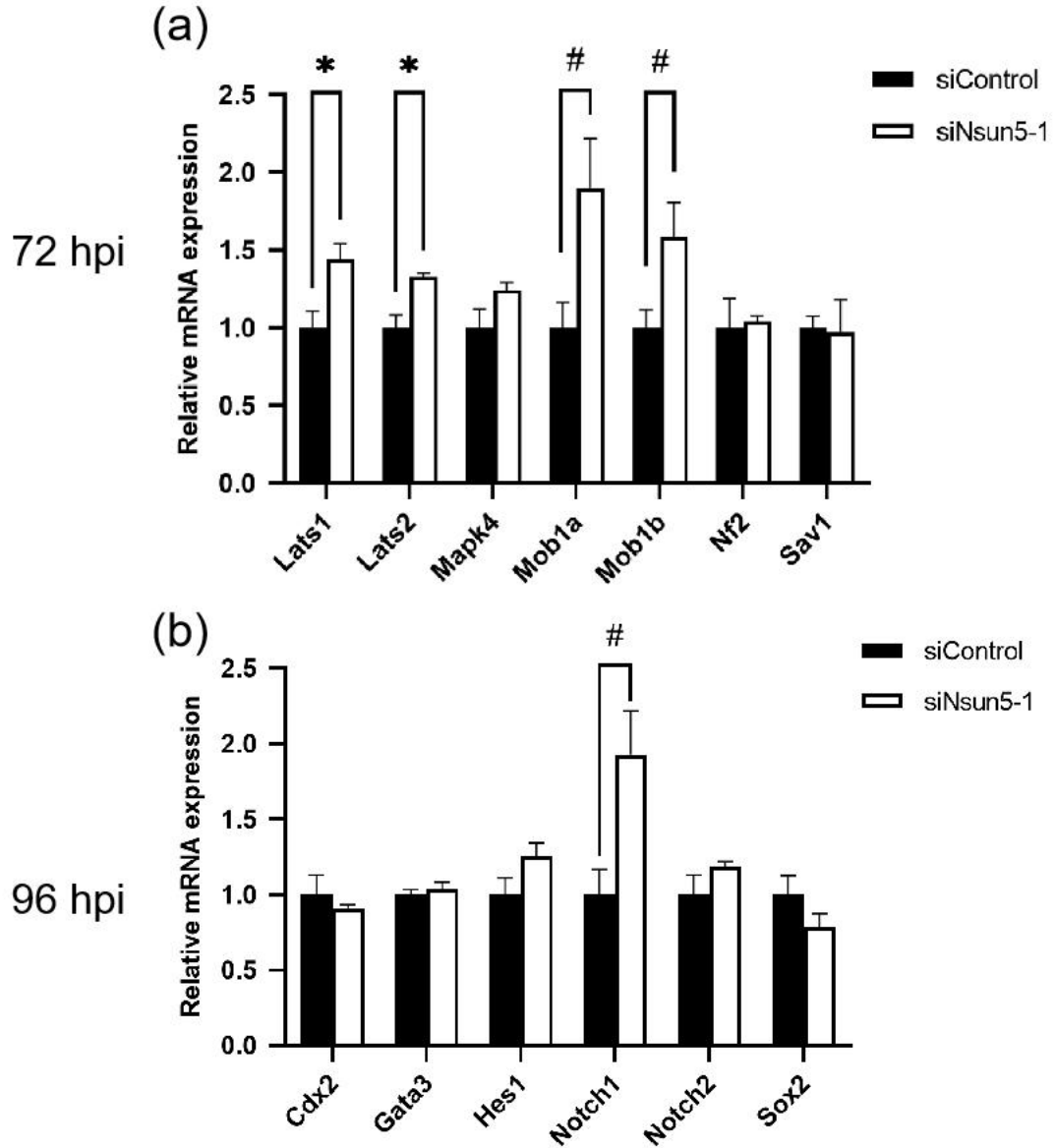


Fig. 2-8. Expression levels of Hippo signaling-related genes at the morula and blastocyst stages.

a Hippo signaling pathway genes were examined using morula stage embryos. **b** Hippo signaling downstream genes were examined using blastocyst stage embryos. Gene expression levels were normalized to *H2afz* as an internal control. Data are expressed as means $\pm$ S.E.M. ($n = 3$). Ten to 13 embryos per replicate in each experimental group were analyzed. # $P < 0.1$, * $P < 0.05$, unpaired t -test.

Chapter 3. Essential role of METTL3 in mitochondrial function and epigenetic regulation during preimplantation development in mice

3.1. Introduction

METTL3 is a key enzyme in m6A RNA modification. As an m6A methyltransferase, it regulates mRNA translation and other cellular functions (87) that are essential for many biological processes, including cell proliferation and differentiation (87, 88). Specifically, METTL3-mediated m6A modification plays pivotal roles in stem cell pluripotency and lineage specification by regulating the stability and translation of key mRNAs (30). In mouse ESCs, METTL3 regulates heterochromatin integrity through interactions with chromatin-modifying complexes (89). In oocytes, maternal *Mettl3* knockdown impairs oocyte maturation and subsequent embryonic development beyond the four-cell stage by affecting mRNA translation efficiency and degradation (73). Recent findings also highlight the interaction of METTL3 with histone-modifying complexes, such as SETDB1 and TRIM28, pointing to a broader epigenetic influence beyond RNA modifications (89).

Histone methylation, particularly of histone H3 lysine 27 and histone H3 lysine 9, plays an essential role in chromatin structure and gene expression regulation during early embryogenesis. H3K27me3, a repressive histone mark, is crucial for maintaining gene silencing and embryonic development (90). In contrast, H3K9me3 is associated with constitutive heterochromatin and gene repression (91). Given the importance of histone modifiers in mouse preimplantation embryos and the

interaction of METTL3 with them, the role of METTL3 in regulating histone methylation marks such as H3K27me3 and H3K9me3 in mouse preimplantation embryos warrants investigation.

The involvement of METTL3 in the Hippo signaling pathway further underscores its critical role in embryonic development. By regulating the m6A methylation of YAP1 and LATS1, components of the Hippo signaling pathway, METTL3 balances cell proliferation and differentiation, processes fundamental to early developmental stages (39). The potential influence of METTL3 on YAP1 activity in preimplantation embryos adds another layer of complexity to our understanding of its multifaceted role during early development.

In addition, recent studies suggest that METTL3 plays a critical role in regulating energy metabolism and mitochondrial function through m6A modification (92, 93). Mitochondria play a crucial role in maintaining cellular homeostasis through the regulation of ROS, ATP production, and calcium signaling. The energy metabolism regulated by mitochondria is essential for preimplantation development because energy demands vary significantly depending on the developmental stage (94). Mitochondria are the primary organelles for ATP production via OXPHOS, which supports the bioenergetic and biosynthetic needs of a developing embryo (95). METTL3-mediated m6A modification of mRNAs encoding mitochondrial proteins influences mitochondrial biogenesis, dynamics, and function (93), ensuring that energy supply aligns with the developmental needs of an embryo (96). METTL3 regulates mitochondrial biogenesis and energy metabolism by modifying peroxisome proliferative-activated receptor gamma coactivator 1 α mRNA (93).

Mitochondrial quality control mechanisms, such as mitochondrial DNA repair,

fusion-fission dynamics, and mitophagy, are vital for maintaining mitochondrial health (97). Redox signaling regulates key mitochondrial functions like nutrient oxidation, OXPHOS, and ROS production (98). These redox-dependent processes influence cellular events such as differentiation, proliferation, and apoptosis (99). Impaired mitochondrial function can disrupt blastocyst development, reduce cell number, and affect fetal and placental growth (100).

While ICM cells primarily rely on glycolysis, TE cells utilize OXPHOS to meet their higher energy demands (101, 102). This metabolic shift is crucial for blastulation and is regulated by the transcription factor TEAD4, which promotes mitochondrial transcription in TE cells (101). The TE cells consumes more oxygen, produces more ATP, and has greater amino acid turnover than the ICM (102). METTL3 might regulate genes linked to mitochondrial function and energy metabolism to meet the energy demands of an embryo, though this remains unproven. Besides, the precise mechanisms underlying this regulation, particularly during early pre-implantation development, are also unclear.

To address these unsolved questions, this study examined the impact of *Mettl3* knockdown on embryonic development, m6A modification, Hippo signaling, histone methylation, mitochondrial function, and comprehensive gene expression in mouse preimplantation embryos.

3.2. Materials and methods

Collection of oocytes, IVF, and embryo culture

ICR female mice (8–12 weeks old; Japan SLC, Shizuoka, Japan) were

superovulated by intraperitoneal injection of 7.5 IU equine chorionic gonadotropin (ASKA Animal Health, Tokyo, Japan) followed 46–48 h later by 7.5 IU human chorionic gonadotropin (ASKA Animal Health). Cumulus-oocyte complexes were collected from the ampullae of the excised oviducts at 14 h after human chorionic gonadotropin injection. Cumulus-oocyte complexes were placed in a 100- μ L droplet of HTF medium supplemented with 4 mg/mL bovine serum albumin (A3311; Sigma-Aldrich, St. Louis, MO) (67). Spermatozoa were collected from the cauda epididymis of 12–18-week-old ICR male mice (Japan SLC) and cultured for at least 1 h in a 100- μ L droplet of HTF medium. After preincubation, the sperm suspension was added to fertilization droplets at a final concentration of 1.0×10^6 cells/mL. At 2 h post-insemination (hpi), morphologically normal fertilized embryos were collected and cultured in potassium simplex optimized medium supplemented with amino acids (68) and 1 mg/mL bovine serum albumin under paraffin oil (Nacalai Tesque, Kyoto, Japan). All incubations were performed at 37°C under 5% CO₂ in air. The embryos were collected or developmentally assessed at each developmental stage after fertilization: early two-cell (24 hpi), late two-cell (36 hpi), four-cell (48 hpi), eight-cell (54 hpi), morula (72 hpi), and blastocyst (96 hpi). All embryos were washed three times in phosphate-buffered saline (PBS) and collected for later experiments.

Microinjection of ASOs

Zygotes (one-cell embryos) were placed in FHM droplets during the microinjection process. ASOs were microinjected into the cytoplasm of zygotes under an inverted microscope (IX73; Olympus, Tokyo, Japan) equipped with a piezo injector (PMAS-CT150; PRIME TECH, Tokyo, Japan) and a micromanipulator (IM-11-2; Narishige, Tokyo, Japan). All fertilized zygotes were microinjected with

either a non-targeting (ASO-Control) or *Mettl3*-targeting ASO (*Mettl3*-ASO) within 3–6 hpi. Approximately 10 pL ASO (5 μ M) solution was injected into the cytoplasm of each zygote. The sequences were as follows:

ASO-Control, GGCTACTACGCCGTCA;

Mettl3-ASO1, GTGGTGTAGCAACTTC;

Mettl3-ASO2, TCAAATCCTGTTCTGC.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Embryos were collected at 24 h (two-cell) after microinjection for RNA extraction to assess knockdown efficiency. Total RNA extraction and cDNA synthesis from embryos were performed using a SuperPrep™ II Cell Lysis & RT Kit for qPCR (TOYOBO, Osaka, Japan). Synthesized cDNA was mixed with specific primers and KOD SYBR qPCR Mix (TOYOBO), followed by RT-qPCR amplification. The protocols for RT-qPCR and measurement of transcript levels were performed as described previously (69), and *H2afz* was used as an internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (70). The following primer sequences were used for RT-qPCR:

H2afz, 5'-GGTAAAGCGTATCACCCCTCG-3' (forward) and
5'-CTTCCCGATCAGCGATTTGTG-3' (reverse);

Mettl3, 5'-CAGGACTCTGGGCACTTGG-3' (forward) and
5'-GCCAGATCTGAGAGGTGGTG-3' (reverse).

Immunofluorescence analysis

Embryos at the late two-cell or morula stage were fixed in 4% paraformaldehyde in PBS at room temperature for 20 min after removal of the zona pellucida with acid Tyrode's solution (pH 2.5). After washing three times in PBS containing 0.3% polyvinylpyrrolidone (K-30; Nacalai Tesque), fixed embryos were treated with 0.5% Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 40 min, and were blocked in PBST containing 1.5% bovine serum albumin, 0.2% sodium azide, and 0.02% Tween 20 (antibody dilution buffer) at room temperature for 1 h. Embryos were incubated with one of the following primary antibodies in antibody dilution buffer at 4°C overnight: anti-METTL3 (1:200 dilution; PA5-41599; Thermo Fisher Scientific, Waltham, MA), anti-m6A (1:1,000 dilution; cat. no. 202 003; rabbit polyclonal, Synaptic Systems, Göttingen, Germany), anti-YAP1 (1:2,000 dilution; DBH1X; Cell Signaling, Danvers, MA), anti-H3K9me3 (1:500 dilution; C15200146; Diagenode, Seraing, Belgium), and anti-H3K27me3 (1:1,000 dilution; C15410069; Diagenode). Embryos were washed three times in antibody dilution buffer and then incubated with an appropriate secondary antibody in antibody dilution buffer (1:500 dilution; Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 594-conjugated goat anti-mouse IgG; Invitrogen, Carlsbad, CA) at room temperature for 1 h. After staining with Hoechst 33342 (Sigma-Aldrich), embryos were mounted on slides in VECTASHIELD mounting medium (Vector Laboratories, Newark, CA) and signals were observed using a fluorescence microscope (BX53 or IX73; Olympus, Tokyo, Japan). The number of analyzed embryos is given in each figure legend. Immunofluorescence intensity in embryos was quantified using ImageJ software (version 1.54f; Java 1.8.0_322, 64-bit) as follows. The region of the embryo in the image was first delineated and the average fluorescence intensity within this region

was measured and the value was termed 'mean intensity'. Concurrently, the area of the region was measured and multiplied by the mean intensity to calculate 'total intensity'.

Assessment of mitochondrial membrane potential

To evaluate mitochondrial membrane potential, a MitoProbe™ JC-1 Assay Kit (M34152; Thermo Fisher Scientific) was utilized. Embryos were incubated with 2.4 μ M JC-1 dye at 37°C for 20 min and subsequently washed twice with KSOM solution. When the dye enters mitochondria, it exhibits a fluorescence shift from green (cytoplasmic JC-1 monomers) to red (mitochondrial JC-1 aggregates) as the mitochondrial membrane becomes more polarized. Fluorescence imaging was performed using an IX73 inverted microscope (Olympus). The acquired images were processed and analyzed using ImageJ software. Mitochondrial membrane potential was quantified by calculating the ratio of red to green fluorescence intensities.

Measurement of mitochondrial ROS

Mitochondrial ROS levels were determined using the MitoSOX™ Mitochondrial Superoxide Indicators for live-cell imaging (M36008; Thermo Fisher Scientific). Embryos were incubated in a 5 μ M MitoSOX™ Red solution at 37°C for 10 min and then washed three times with KSOM solution. Fluorescent signals were captured using an IX73 inverted microscope (Olympus). Fluorescence intensity was quantified using ImageJ software.

RNA sequencing

Morula stage embryos ($n = 10$) in the ASO-Control and *Mettl3*-ASO groups were collected for RNA sequencing (RNA-seq) library construction at 72 hpi. Two biological replicates were obtained from each group. Polyadenylated RNA-seq libraries were prepared by using a SMART-Seq v4 PLUS Kit (Clontech, Mountain View, CA). Indexed RNA-seq libraries were sequenced using an Illumina NovaSeq X Plus sequencer (150-bp paired-end). Raw RNA-seq reads were trimmed to remove low-quality bases and adapter sequences using fastp (ver. 0.20.1) (103). The resulting sequence reads were mapped to the mm10 mouse genome using STAR aligner software (ver. 2.7.10.a), and only uniquely mapped reads were used for subsequent processing. Gene expression levels were calculated using RSEM (ver. 1.3.3) and differentially expressed genes (DEGs) were analyzed with the DESeq2 package (ver. 1.38.3). DEGs were defined as those with $|\log_2 \text{fold change}| > 1$ and adjusted P -value < 0.05 . To generate volcano plots and polynomial correlation analysis, the RNA-seq data were analyzed and visualized in R (<http://www.R-project.org>). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was also carried out in R.

3.3. Statistical analysis

Developmental rates, data for RT-qPCR, and immunofluorescence intensity for METTL3 and m6A were assessed using one-way analysis of variance followed by Fisher's LSD test. Cell number, JC-1 staining, MitoSOX Red staining, and other immunofluorescence intensity data were analyzed using Student's t -test for pairwise comparisons or a nonparametric Mann-Whitney U test depending on the

characteristics of the data, as described below. The nonparametric Mann-Whitney U test was used when the majority of normality tests, such as D'Agostino & Pearson, Anderson-Darling, and Shapiro-Wilk, showed that the data were not normally distributed ($P < 0.05$).

3.4. Results

***Mettl3*-ASO2 efficiently knocks down METTL3 and m6A**

Two ASOs targeting *Mettl3* were used to suppress its expression. RT-qPCR was carried out at 24 h after microinjection. *Mettl3*-ASO1 and -ASO2 both significantly reduced *Mettl3* mRNA levels (Fig. 3-2a). The effects of the ASOs on METTL3 protein levels and m6A modification were confirmed in mouse embryos at 30 h after microinjection using immunofluorescence analysis. There were no discernible alterations in the amounts of m6A or METTL3 protein in embryos injected with *Mettl3*-ASO1 compared to the control group (Fig. 3-2b). In contrast, embryos injected with *Mettl3*-ASO2 showed a significant reduction in METTL3 protein levels and tended to have reduced m6A levels (Fig. 3-2c), but not significantly ($P < 0.1$).

***Mettl3* knockdown impairs preimplantation embryonic development in mice**

Embryos in the *Mettl3*-ASO1 group failed to develop to the morula stage, indicating early developmental arrest (Fig. 3-3a and b). Meanwhile, the *Mettl3*-ASO2 group showed a significant inhibition of blastocyst formation, with only 11.5% of embryos forming blastocysts compared to 89.4% in the control group (Fig. 3-3a and b). *Mettl3*-ASO2 was chosen for the following experiments also considering its ability

to reduce protein expression and m6A levels (Fig. 3-2c).

***Mettl3* knockdown reduces cell number and YAP1 expression**

Anti-YAP1 antibody and Hoechst staining was conducted on embryos at 72 hpi. A decrease in cell number was observed in the *Mettl3* knockdown group compared to the control group. Precisely, the average cell number in the control group embryos was 18, while the knockdown group embryos exhibited a significantly lower average of 14 cells (Fig. 3-4a and b). The mean fluorescence intensity of YAP1 was significantly reduced by 19% in the knockdown group compared to the control group (Fig. 3-4c and d). *Mettl3* knockdown did not interfere with the nuclear translocation of YAP1 (Fig. 3-5a and b). Although the number of YAP1-positive nuclei was lower in the knockdown group embryos than in the control group (Fig. 3-5a), this was attributable to the reduced total cell number in the knockdown group. The percentage of YAP1-positive nuclei relative to total cell number was not significantly different between both groups (Fig. 3-5b). Nevertheless, *Mettl3* knockdown resulted in a decrease in YAP1 expression levels within the embryos.

***Mettl3* knockdown disrupts histone modification**

The levels of two important histone modifications, H3K27me3 and H3K9me3, in the nuclei of morula-stage embryos were measured using immunofluorescence staining. Representative images of the nuclear staining of H3K27me3 are shown in Figure 3-6a, highlighting the differences in fluorescent signals across the groups. The average fluorescence intensity of H3K27me3 within the nucleus of embryos was 15% lower in the *Mettl3* knockdown group than in the control group (Fig. 3-6a and b).

These data suggest decreased levels of H3K27me3 in response to *Mettl3* knockdown. In contrast to H3K27me3, the mean nuclear fluorescence intensity for H3K9me3 did not differ significantly between both groups (Fig. 3-7a and b).

***Mettl3* knockdown increases mitochondrial membrane potential and ROS**

Mettl3 knockdown significantly increased the mitochondrial membrane potential in mouse morula-stage embryos, as determined by the JC-1 assay. More precisely, a 15% increase in the red-to-green fluorescence ratio was indicative of raised mitochondrial polarization (Fig. 3-8a). Furthermore, the MitoSOX Red assay revealed that embryos in the knockdown group had higher levels of mitochondrial ROS than the control group, indicating greater oxidative stress (Fig. 3-8b).

Impact of *Mettl3* knockdown on GO and KEGG pathway enrichment analysis

A total of 276 DEGs were identified in preimplantation embryos subjected to *Mettl3* knockdown, as compared to control embryos ($P < 0.05$). Among these DEGs, 108 were downregulated, whereas 168 were upregulated in the *Mettl3* knockdown group relative to the control group (Fig. 3-9a).

GO and KEGG pathway enrichment analysis was performed on the DEGs. GO analysis classified the enriched pathways into the Biological Process, Cellular Component, and Molecular Function categories. In the Biological Process category, nucleotide metabolic processes and RNA splicing were the most prominent, while the Cellular Component category highlighted significant enrichment in mitochondrial and nuclear components. The Molecular Function category underscored the crucial role of

isomerase activity, particularly peptidyl-prolyl cis-trans isomerase, in the molecular alterations observed (Fig. 3-9b). KEGG pathway analysis identified the most significantly enriched pathways, including those related to “Biosynthesis of amino acids” and “Carbon metabolism,” emphasizing the major importance of metabolic processes in the observed changes (Fig. 3-9c).

3.5. Discussion

This study investigated the role of METTL3 during early preimplantation development in mice through targeted knockdown of *Mettl3* using ASOs. The findings reveal significant insights into the functions of METTL3 in cellular processes such as gene expression, cell proliferation, mitochondrial function, histone modification, and key signaling pathways. Firstly, both *Mettl3*-ASO1 and -ASO2 effectively reduced *Mettl3* mRNA levels, but only *Mettl3*-ASO2 significantly decreased METTL3 protein levels. *Mettl3*-ASO2 also tended to reduce m6A levels in embryos. This outcome suggests that *Mettl3*-ASO2 is more efficient in target-specifically disrupting the METTL3-mediated modification of m6A. These findings support our hypothesis that METTL3 plays a critical role in maintaining m6A levels, which in turn affects downstream cellular processes crucial for early embryonic development.

The inefficacy of *Mettl3*-ASO1 in reducing METTL3 protein and m6A levels, despite lowering mRNA levels, may point toward post-transcriptional regulatory mechanisms. This discrepancy could indicate that METTL3 protein stability is maintained through alternative pathways, such as feedback loops or compensatory mechanisms, which warrant further investigation.

Mettl3 knockdown impaired preimplantation embryonic development, with *Mettl3*-ASO1-treated embryos arresting before the morula stage and *Mettl3*-ASO2-treated embryos also exhibiting a drastic reduction in the morula stage. These results in mice are in line with previous studies that found *Mettl3* knockdown results in low blastocyst formation rates, decreased expression of important transcription factors, and developmental arrest in goat embryos (104), further supporting the conserved role of METTL3 in early embryogenesis across species. The significant decrease in blastocyst formation in *Mettl3*-ASO2-treated embryos underlines the critical function of METTL3 in early embryogenesis. Additionally, the reduced number of cells in *Mettl3* knockdown embryos suggests that METTL3 influences cell proliferation.

Interestingly, while YAP1 expression was decreased in the *Mettl3* knockdown group, its nuclear translocation remained unaffected. This suggests that METTL3 impacts overall YAP1 expression rather than its localization, hinting at complex regulatory mechanisms that may involve the m6A modification of YAP1-related transcripts. Given the role of YAP1 in cell proliferation in early embryos, its reduced expression may partly explain the observed decrease in cell number.

Mettl3 knockdown also disrupted histone modifications, specifically reducing H3K27me3 levels, but not affecting H3K9me3. The decrease in H3K27me3 highlights a potential link between METTL3 and epigenetic regulation, consistent with previous reports that METTL3 is involved in chromatin remodeling (89), which suggests that its role in maintaining H3K27me3 may also be mediated through interactions with histone-modifying complexes. However, the unchanged levels of H3K9me3 suggest selective effects on specific histone modifications, raising interesting questions about the mechanisms by which METTL3 influences chromatin

structure.

Mitochondrial function in *Mettl3* knockdown embryos was altered, as indicated by an increase in mitochondrial membrane potential and elevated levels of ROS. While mitochondrial activity is essential for energy production, both excessive and insufficient metabolic activity can be detrimental to embryonic development, highlighting the need to maintain mitochondrial function within an optimal range (105). Previous studies have shown that arrested mouse two-cell embryos exhibit increased mitochondrial membrane potential compared to non-arrested embryos, and in human embryos, this heightened mitochondrial activity is linked to greater levels of fragmentation (106). Moreover, elevated ROS levels cause oxidative stress, which can lead to embryonic arrest, DNA damage, and abnormal fetal development (107). These results align with our findings, suggesting that the mitochondrial hyperactivity and increased ROS observed in *Mettl3* knockdown embryos likely contribute to the impaired developmental outcomes, underscoring the delicate balance required for proper mitochondrial function during early embryogenesis.

In terms of gene expression, transcriptomic analysis identified 276 DEGs in *Mettl3* knockdown embryos. GO and KEGG pathway enrichment analysis revealed significant alterations in pathways related to metabolic processes, RNA splicing, and mitochondrial components. These findings emphasize the role of METTL3 in regulating metabolic and RNA processing pathways during early development. The significant enrichment of metabolic pathways, particularly amino acid biosynthesis and carbon metabolism, underscores the impact of *Mettl3* knockdown on fundamental cellular processes essential for embryonic growth and differentiation.

While this research provides significant insights into the role of METTL3 during

early development, several limitations should be acknowledged. First, the exact mechanisms through which METTL3 influences histone modifications and YAP1 expression remain unclear. Although this study demonstrated reductions in H3K27me3 levels and YAP1 expression, the specific pathways and m6A-dependent mechanisms involved in these changes require further investigation.

This study shows the essential role of *Mettl3* in mouse preimplantation embryonic development. *Mettl3* knockdown disrupts multiple essential processes in mouse embryos, leading to impaired blastocyst formation, reduced cell number, and altered mitochondrial function. The critical role of *Mettl3* in mouse preimplantation embryonic development points to potential therapeutic strategies for addressing reproductive disorders and improving fertility.

Figures

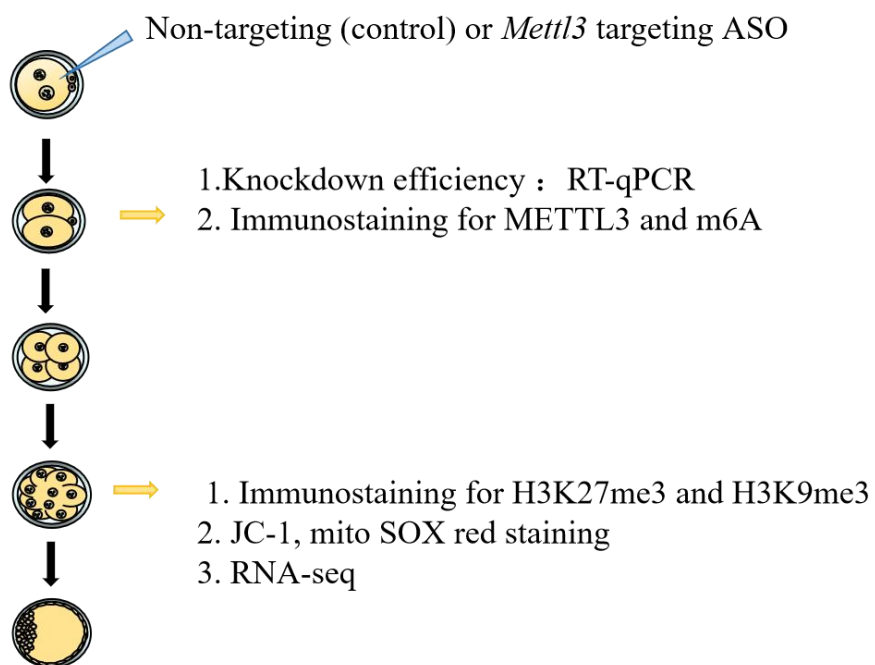


Fig. 3-1. Experimental workflow

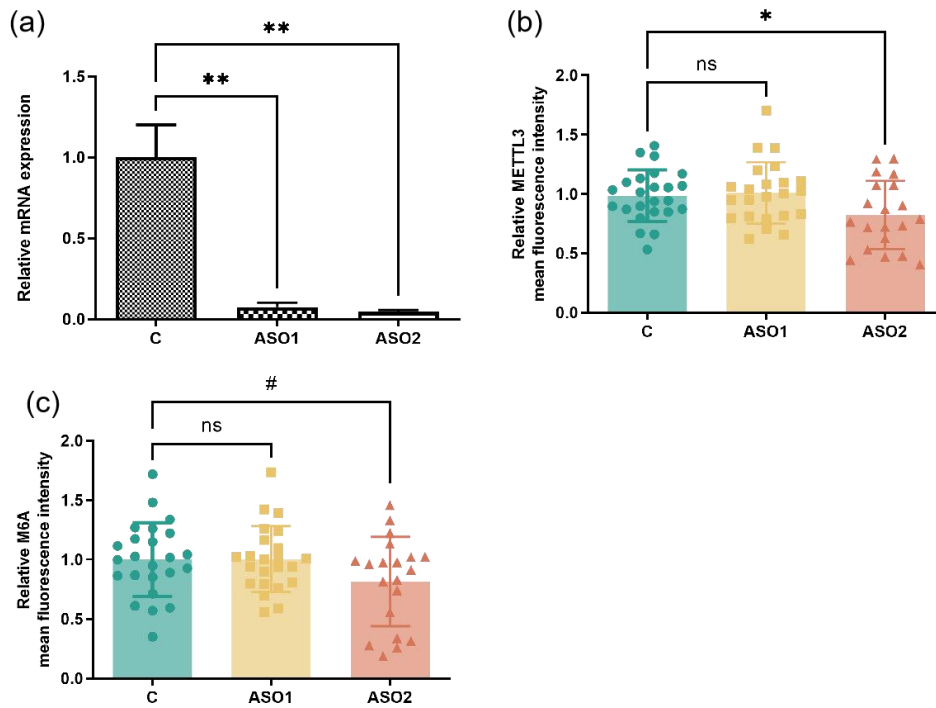


Fig. 3-2. Knockdown efficiency of ASO treatment and effect on m6A levels. **a** Knockdown efficiency of ASO treatment at the two-cell stage (three replicates). Fourteen to 22 embryos per replicate in each experimental group were analyzed. **b** Relative METTL3 protein levels. **c** Relative m6A levels. All fertilized zygotes were microinjected with ASO-Control or two different ASOs targeting *Mettl3* within 3–6 hpi. Embryos were subjected to RT-qPCR or immunostaining to assess knockdown efficiency at 24 or 30 h after injection, respectively. Data are expressed as the mean $\pm$ standard error of the mean. ASO-Control, $n = 24$; *Mettl3*-ASO1, $n = 23$; *Mettl3*-ASO2, $n = 20$. Statistical analysis was performed using one-way analysis of variance followed by Fisher's LSD test. Not significant (ns) $P > 0.1$, # $P < 0.1$, * $P < 0.05$, ** $P < 0.01$. Dots in (b) and (c) represent each embryo.

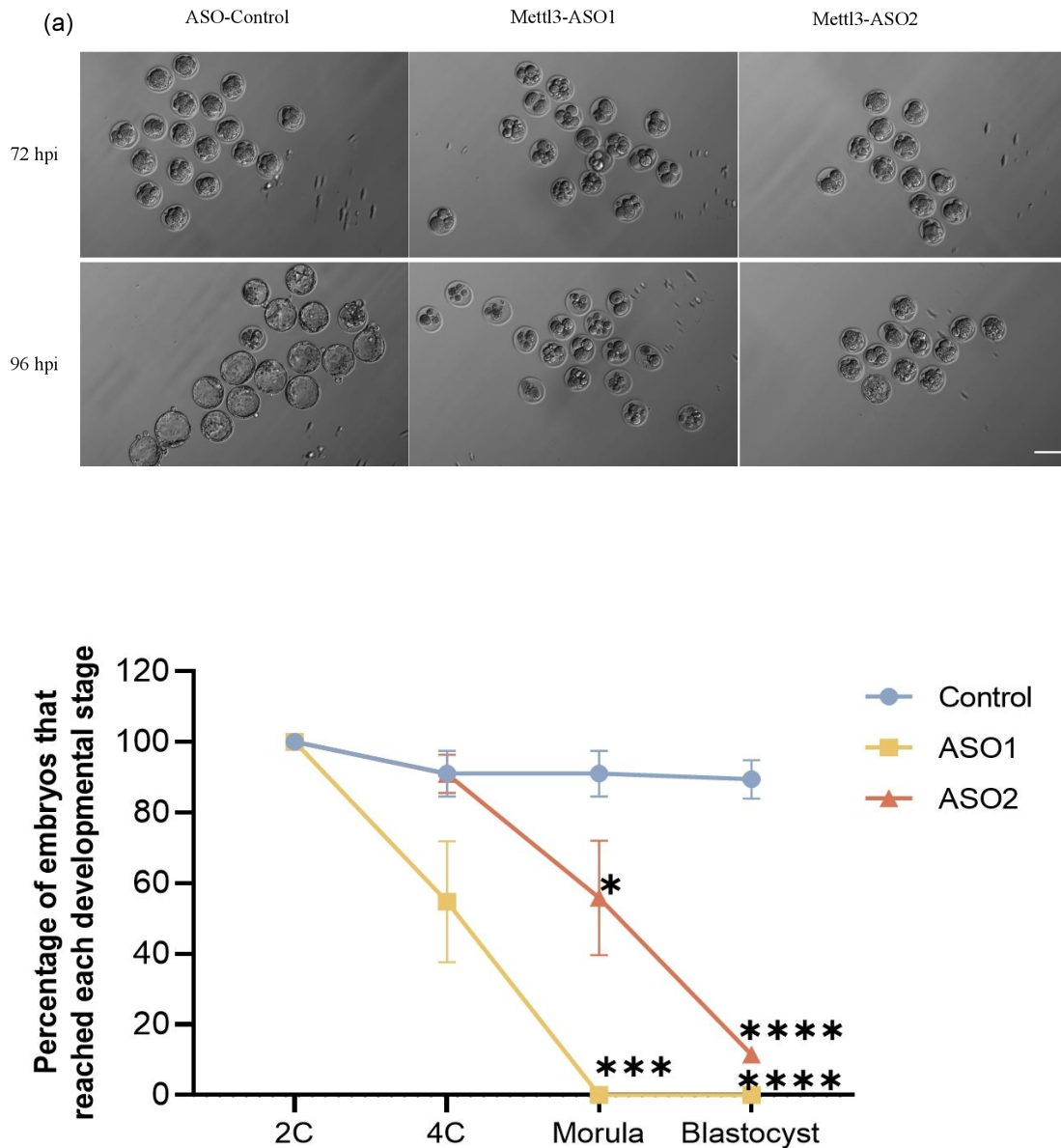


Fig. 3-3. Effects of *Mettl3* knockdown on mouse preimplantation embryonic development. **a** Morphology of mouse embryos after ASO-Control or *Mettl3*-ASO microinjection. Scale bar, 100 μ m. Embryos were photographed at 72 and 96 hpi. **b** Percentage of embryos that reached each developmental stage. Zygotes were cultured *in vitro* until 96 hpi and the percentage of embryos that reached each developmental stage was quantified. Data are expressed as the mean $\pm$ standard error of the mean (three replicates). ASO-Control, $n = 53$; *Mettl3*-ASO1, $n = 51$; *Mettl3*-ASO2, $n = 50$.

$*P < 0.05$, $***P < 0.001$, $****P < 0.0001$, one-way analysis of variance followed by Fisher's LSD test.

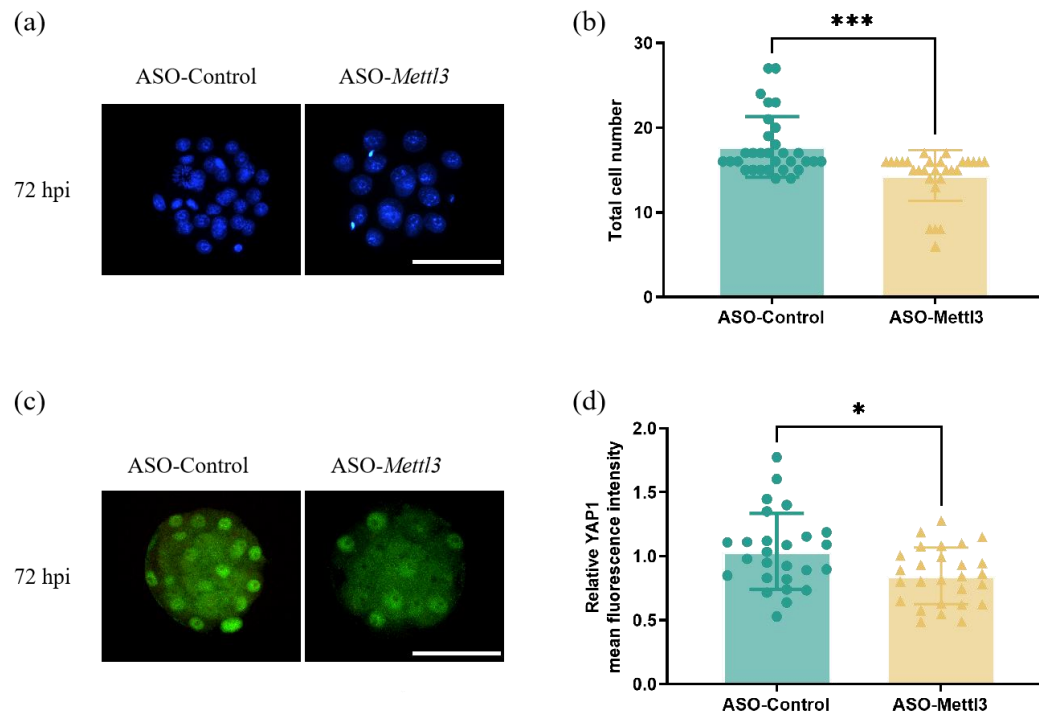


Fig. 3-4. Effects of *Mettl3* knockdown on total cell number and YAP1 expression in embryos. **a** Images of Hoechst-stained mouse preimplantation embryos at the morula stage (72 hpi) used for the assessment of total cell number. Scale bar, 100 μ m. **b** Total cell number of mouse morula stage embryos. ASO-Control, $n = 31$; *Mettl3*-ASO2, $n = 28$. *** $P < 0.001$, unpaired t -test. **c** Immunostaining images of mouse preimplantation embryos at the morula stage (72 hpi) to assess YAP1 expression levels. **d** Mean fluorescence intensity of YAP1 throughout the embryo at the morula stage. ASO-Control, $n = 31$; *Mettl3*-ASO2, $n = 28$. * $P < 0.05$, unpaired t -test.

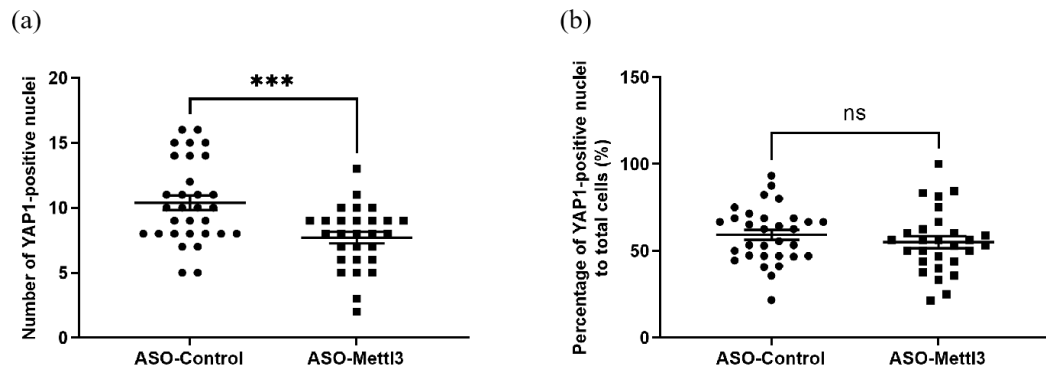


Fig. 3-5. Effects of *Mettl3* knockdown on YAP1 nuclear translocation. **a** Number of cells that exhibited YAP1 nuclear localization at the morula stage. **b** Ratio of nuclear YAP1-positive cells to total cell number at the morula stage. ASO-Control, $n = 31$; *Mettl3*-ASO2, $n = 28$. Not significant (ns) $P > 0.1$, *** $P < 0.001$, unpaired t -test.

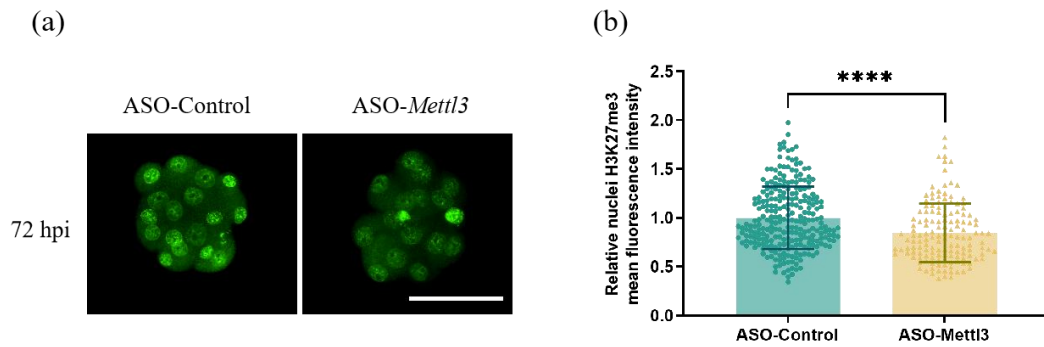


Fig. 3-6. Effects of *Mettl3* knockdown on H3K27me3 levels at the morula stage. **a** Representative images of H3K27me3 immunostaining. Scale bar, 100 μ m. **b** Quantification of relative nuclear immunofluorescence intensity of H3K27me3. Each dot represents one nucleus. ASO-Control, number of embryos = 19, number of nuclei = 262; *Mettl3*-ASO2, number of embryos = 15, number of nuclei = 144. **** $P < 0.0001$, Mann-Whitney U test. Normality tests (D'Agostino & Pearson, Anderson-Darling, and Shapiro-Wilk) indicated that the data were not normally distributed ($P < 0.05$); therefore, the nonparametric Mann-Whitney U test was used.

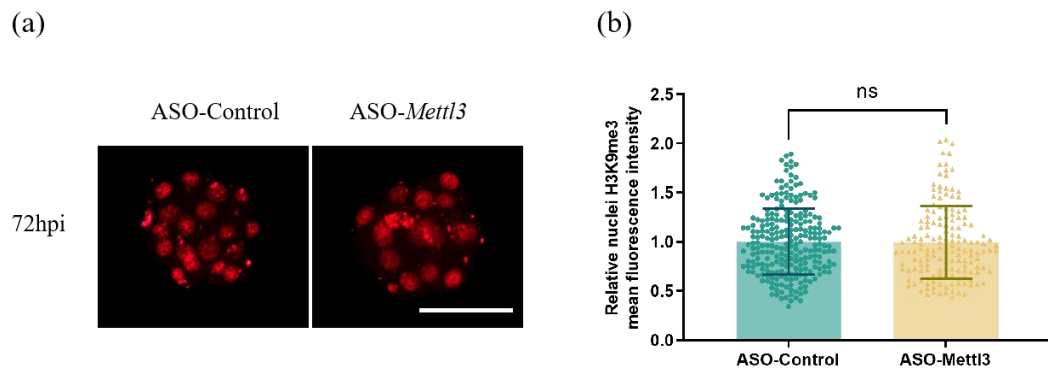


Fig. 3-7. Effects of *Mettl3* knockdown on H3K9me3 levels at the morula stage. **a** Representative images of H3K9me3 immunostaining. **b** Quantification of relative nuclear immunofluorescence intensity of H3K9me3. Each dot represents one nucleus. ASO-Control, number of embryos = 19, number of nuclei = 246; *Mettl3*-ASO2, number of embryos = 15, number of nuclei = 155. Not significant (ns) $P > 0.1$, Mann-Whitney U test. Normality tests (D'Agostino & Pearson, Anderson-Darling, and Shapiro-Wilk) indicated that the data were not normally distributed ($P < 0.05$); therefore, the nonparametric Mann-Whitney U test was used.

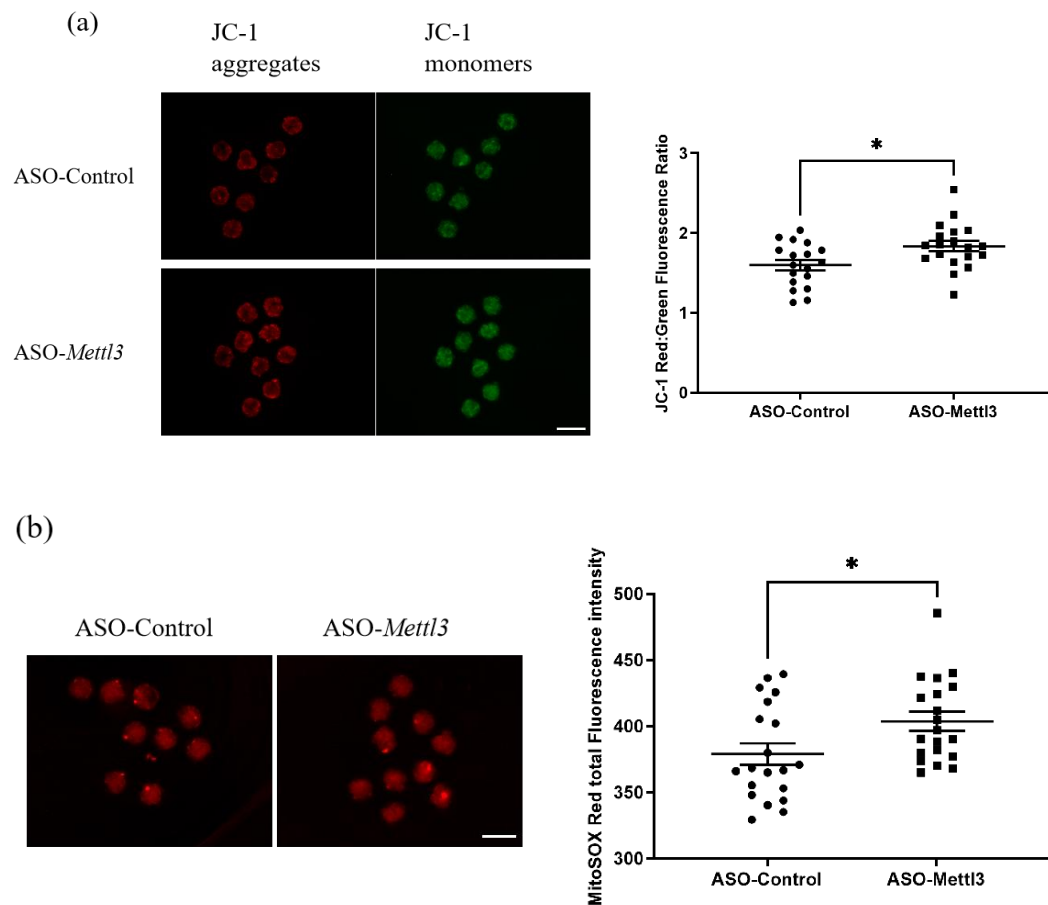
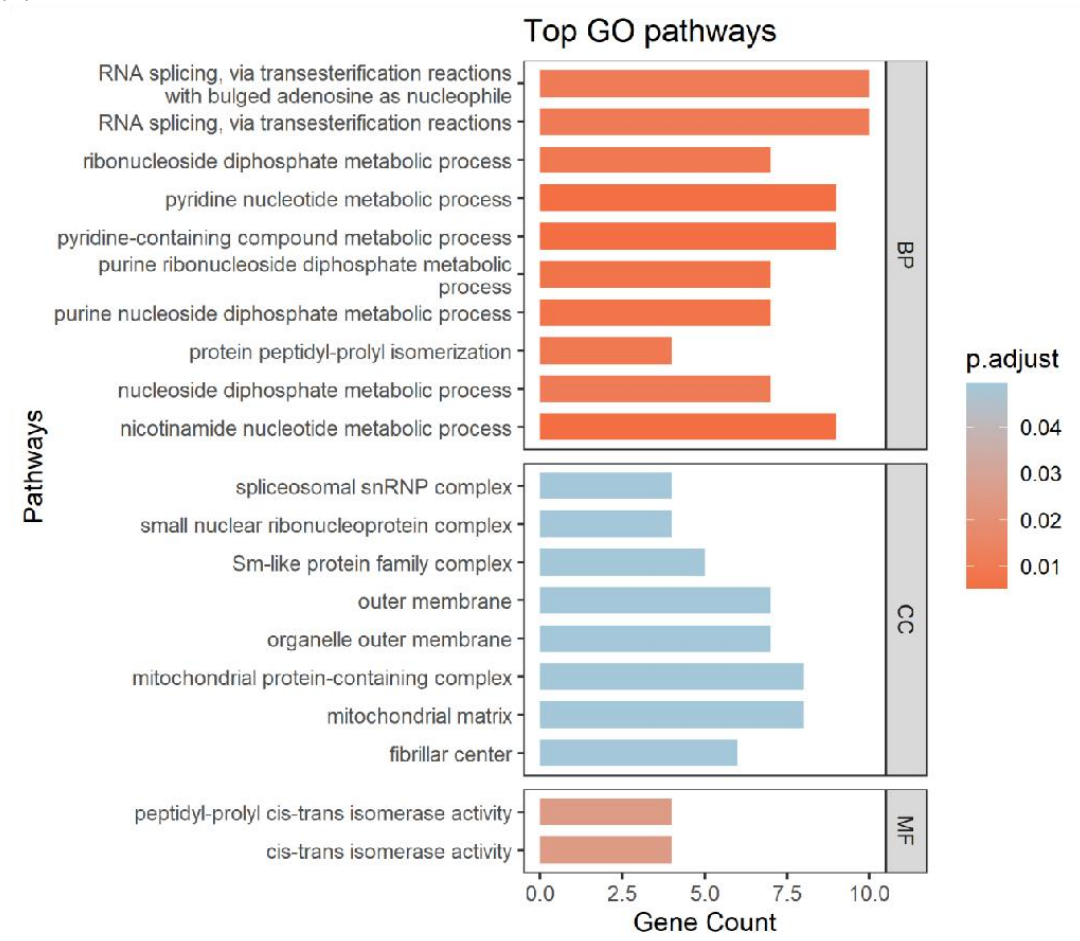


Fig. 3-8. Mitochondrial analysis. **a** Mitochondrial membrane potential in the embryos of the control and Mettl3 knockdown groups at the morula stage. Left: Representative images of JC-1 staining. Right: measuring the ratio of red (JC-1 aggregates) to green (JC-1 monomers) fluorescence to determine mitochondrial membrane potential. Scale bar 100 μ m. ASO-Control, $n = 18$; Mettl3-ASO2, $n = 19$. **b** ROS levels in morula stage embryos. Left: representative images of MitoSOX Red staining. Right: quantification of intramitochondrial ROS levels. Scale bar, 100 μ m. ASO-Control, $n = 20$; Mettl3-ASO2, $n = 20$. $*P < 0.05$, unpaired t-test.

(a)



(b)



(c)

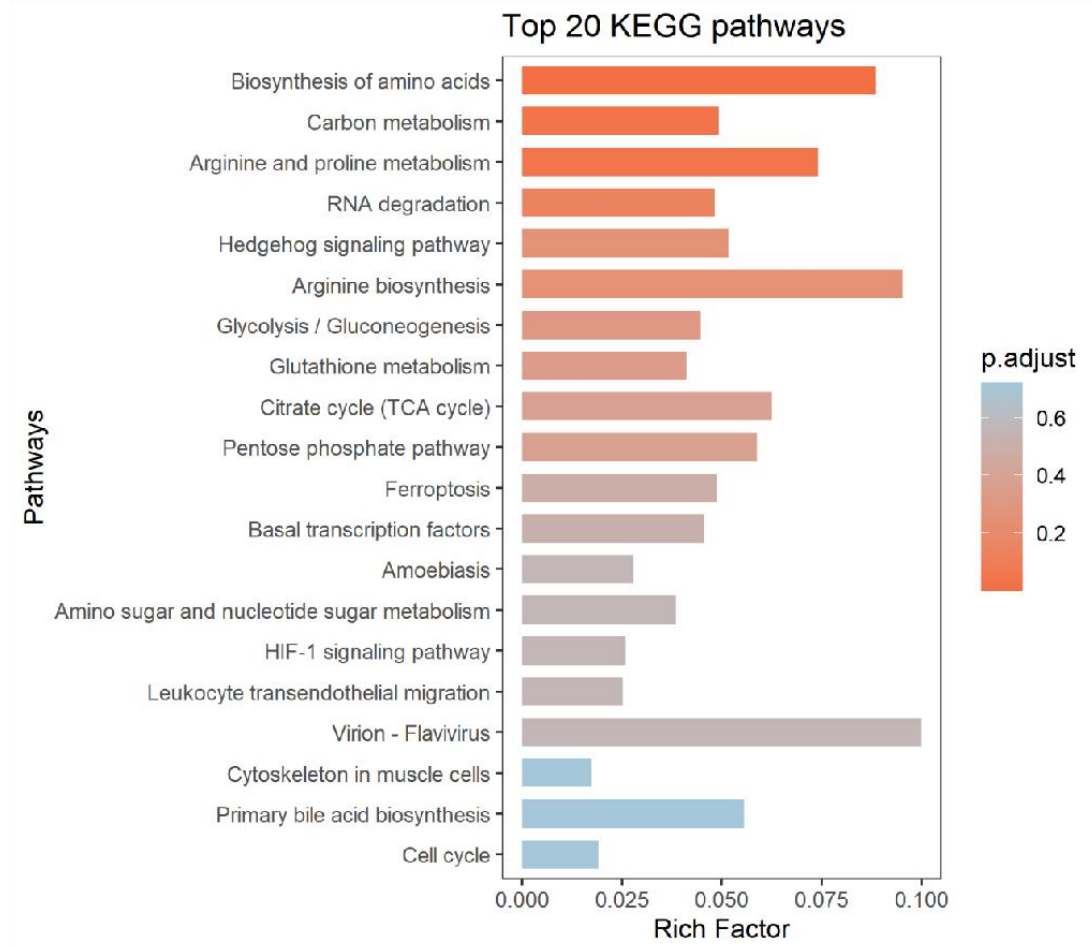


Fig. 3-9. RNA-seq analyses in *Mettl3* knockdown embryos. **a** Volcano plot of RNA-seq data comparing gene expression of *Mettl3* knockdown embryos with control group embryos at the morula stage. The *x*-axis represents the log₂ fold change, and the *y*-axis shows the -log₁₀ adjusted *P*-value. Red dots indicate significantly upregulated genes, green dots represent significantly downregulated genes, and gray dots indicate non-significant genes. The blue dot highlights *Mettl3*. Significance thresholds are set at |log₂ fold change| > 1 and adjusted *P*-value < 0.05. **b** GO analysis of DEGs. The most significant GO terms across the Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) branches are shown, with adjusted *P*-values. The number of GO terms are 10, 8, and 2 for BP, MF, and CC, respectively. **c** KEGG pathway analysis of the DEGs. Top 20 enriched KEGG

pathways of the DEGs. which is the ratio of the number of DEGs mapped to a pathway to the total number of genes in that pathway. The color gradient indicates the adjusted *P*-value (p.adjust), with red representing higher significance (lower p.adjust) and blue indicating lower significance (higher p.adjust). Pathways are ranked by enrichment significance.

Chapter 4. General summary

The current study explores the roles of two distinct RNA modification enzymes, NSUN5 and METTL3, to elucidate their contribution to preimplantation embryonic development in mice. By knocking down Nsun5 and Mettl3 via targeting siRNAs or ASOs, this research uncovers how these RNA modification enzymes regulate fundamental cellular processes, including cell proliferation, apoptosis, metabolic activity, and epigenetic modifications. In this discussion, I compare the effects of Nsun5 and Mettl3 knockdown, evaluate their contribution to embryonic development, and integrate these findings with the broader context of existing literature.

NSUN5, responsible for m5C modification, and METTL3, catalyzing m6A modification, both play pivotal roles in regulating gene expression and cellular differentiation (108). In this study, Nsun5 knockdown led to reduced cell proliferation, increased apoptosis, and abnormal differentiation in preimplantation embryos. These phenotypes were similar to those observed with Mettl3 knockdown, where blastocyst formation was significantly reduced and cell numbers decreased. In both cases, they appear to act as key modulators of mouse early development, albeit through different molecular mechanisms.

The knockdown of Nsun5 specifically highlighted its role in maintaining cell proliferation. this finding aligns with studies that demonstrate NSUN5's role in regulation of cell cycle (47). Similarly, Mettl3 knockdown resulted in decreased cell numbers, suggesting that m6A methylation of transcripts involved in the cell cycle is critical for ensuring normal embryonic proliferation. Despite this similarity in phenotypes, the underlying pathways appear to diverge. For instance, Nsun5

knockdown perturbs Hippo signaling by decrease the nuclear translocation of YAP1 with no effect on YAP expression. While Mettl3 knockdown decrease the YAP expression with no effect on YAP1 nuclear translocation.

METTL3's impact on epigenetic regulation was directly observed through histone modifications, particularly the reduction in H3K27me3 levels. This histone modification is known to maintain gene silencing during development and is essential for proper embryonic differentiation. While H3K9me3 levels remained unaffected in METTL3 knockdown embryos, the reduction in H3K27me3 suggests that m6A methylation of key transcripts influences chromatin remodeling, possibly through the regulation of Polycomb group proteins (41). This supports previous findings that METTL3 is involved in both transcriptional and post-transcriptional regulation, affecting gene expression on multiple levels (109).

Nsun5 knockdown embryos exhibited increased apoptosis. Apoptosis is a feature that is critical to understanding the role of RNA modifications in early development. For Nsun5, the increase in apoptosis could be linked to the altered YAP1 activity through Hippo signaling. YAP1 is a key transcriptional co-activator in the Hippo pathway and regulates cell proliferation and apoptosis. The knockdown of Nsun5 resulted in reduced YAP1 nuclear translocation, which likely led to an imbalance in pluripotent and TE cell lineages. This is supported by previous studies demonstrating that the Hippo pathway is essential for lineage specification during preimplantation development (110, 111).

Mettl3 knockdown resulted in increased ROS level, which may be a marker of early apoptosis (112, 113). The mechanistic basis also differs. METTL3 is responsible for m6A modification of thousands of mRNAs, including those involved in cell cycle

regulation and apoptosis control. The disruption of m6A marks destabilizes these mRNAs, leading to widespread dysregulation of gene expression. YAP1 nuclear localization was not affected in METTL3 knockdown embryos, suggesting that m6A-mediated apoptosis is independent of Hippo signaling. Instead, Gene ontology (GO) and KEGG pathway enrichment analyses highlighted metabolic processes, RNA splicing, and mitochondrial components as significantly altered aspects. These findings emphasize METTL3's role in regulating metabolic and RNA processing pathways during early development .

Interestingly, while Mettl3 knockdown led to mitochondrial hyperactivity, the embryos failed to develop beyond the blastocyst stage, suggesting that excessive metabolic activity can be just as detrimental as insufficient metabolism. A previous study has shown that hyperactivity of metabolic stalling are linked to embryonic arrest (106), supporting the idea that RNA modifications such as m5C and m6A help maintain an optimal metabolic balance during development (105). These findings add a new dimension to the growing understanding of how RNA methylation influences cellular metabolism in early-stage embryos, with potential implications for understanding metabolic disorders and infertility.

My findings are consistent with previous studies showing the critical role of m6A and m5C modifications in embryonic development, yet provide additional insights into their specific molecular functions. For example, earlier research has established that METTL3 is essential for m6A methylation in mammalian cells and that m6A is a key regulator of mRNA stability and translation (114). This study extends this understanding by demonstrating that m6A also regulates histone modifications, specifically H3K27me3, thereby affecting chromatin structure and gene expression. In contrast, although NSUN5's role in m5C modification has been documented in oocyte

development (76), my study reveals its importance in modulating Hippo signaling and YAP1 activity in preimplantation embryos. This distinction between the regulatory networks governed by m5C and m6A highlights the diversity of RNA modifications in controlling different aspects of early development.

In summary, this research underscores the significance of RNA modification enzymes NSUN5 and METTL3 in regulating key processes during preimplantation embryogenesis. By comparing the effects of Nsun5 and Mettl3 knockdown, I show that both RNA methylation pathways are crucial for maintaining cell proliferation, differentiation, apoptosis, and epigenetic regulation. However, these modifications act through distinct molecular mechanisms, with m5C primarily influencing transcriptional regulation and Hippo signaling, while m6A affects both post-transcriptional processes and histone modifications. This suggests that RNA methylation is a multifaceted regulatory system that modulates embryonic development at multiple levels, from RNA stability to chromatin organization and metabolic control.

The implications of this research are profound, particularly in the context of reproductive medicine and embryo development disorders. Targeting RNA modifications could offer new therapeutic avenues for addressing issues related to infertility or embryo viability. However, future studies are required to fully elucidate the precise molecular mechanisms by which these modifications regulate early development and to explore their potential as therapeutic targets.

In conclusion, Nsun5 and Mettl3 playing critical roles in maintaining cellular and metabolic balance. These findings contribute to a deeper understanding of RNA modifications and their broader implications for developmental biology and medical

research.

Acknowledgements

First of all, I would like to express my deepest gratitude to my advisor, Dr. Shuntaro Ikeda, and Dr. Naojiro Minami, who provided valuable guidance, continued support, and encouragement throughout my entire Ph.D. pursuit. The knowledge and wisdom they have imparted to me are truly instrumental in my growth as a better researcher and scholar. I would especially like to thank Dr. Shinnosuke Honda for his insightful comments and generous support.

I would also like to thank Dr. Zeng Yiren, Ms. Wang Haoxue, Ms. Gao Yuwei, Ms. Chen Xinyao, and others in the Laboratory of Reproductive Biology who have contributed to a positive and encouraging atmosphere. Their support and friendship have always inspired me during both hard times and fruitful times. My special thanks go to Ms. Chen Xinyao for helping share the experimental workload of my second study.

My husband, Mr. Zhang Yuhui, gave me a lot of psychological support and was concerned about my every step in the process of PhD study. I regard his encouragement during the most difficult period as a great help to me in maintaining my concentration and enthusiasm in studying in daily life.

I am deeply indebted to my family for their unconditional love, patience, and confidence in me. My parents, Mr. Liu Guixuan and Mrs. Lu Xiaoping have been the greatest source of strength for me, and to them, I owe my achievements to their endless support. My siblings, Ms. Liu Hui and Mr. Liu Guanhong, I would also like to thank them for their continued encouragement and for knowing ways to make me smile. Their energy is just a really comforting and joyful positive aspect of my journey.

Lastly, I will thank all those who have formed part of this journey, supporting me in any way.

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