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RESEARCH ARTICLE

Rapamycin Alters Oxidative Stress and Tumor-Associated Protein Profiles in Chicken Embryonic Stem Cells

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ABSTRACT

Embryonic stem cells (ESCs) of chicken undergo a process of controlled autophagy to maintain their stemness during in vitro culture. This controlled autophagy may contribute to differentiation of ESCs to primordial germ cells (PGCs). Our previous study elucidated the role of rapamycin in activating autophagy in chicken ESCs. Treatment of ESCs with rapamycin resulted in the upregulation of autophagy-related proteins, including MAPLC3A and MAPLC3B. In the present study, an extensive proteomic analysis of chicken embryonic stem cells (ESCs) following treatment with rapamycin was conducted. Liquid chromatography mass spectrometry analysis was performed on three biological replicates of both rapamycin-treated (ESC-Rapa) and untreated ESCs (ESC-Blank), identifying 4,495 proteins. Of these, 126 were found to be upregulated, and 325 downregulated based on log2FC (FC≥1.5). Furthermore, a total of 4,044 proteins exhibited non-significant (P>0.05) regulation in both the ESC-Blank and ESC-Rapa groups. The downregulated proteins were predominantly associated with the activation of tumors in various organs throughout the body as determined in several studies. Several tumor-promoting proteins were found to be significantly (P<0.05) downregulated, including HDAC2, YWHAG, HMGB3, PDCD10, POFUT1, VDAC2, and ovoinhibitor. These results suggest that rapamycin may promote autophagy, which could be attributed to the downregulation of tumor-activating genes and proteins. However, further research is needed to determine the role of rapamycin in tumor suppression and the development of chicken embryonic stem cells.

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INTRODUCTION

Chicken embryonic stem cells (ESCs) are an ideal system for studying the processes of stem cell propagation and differentiation. A substantial body of research has revealed that embryonic stem cells manifest elevated levels of pluripotency genes, which are concomitant with the proliferation of various types of cancer cells (Ling *et al.,* 2012; Rios *et al.,* 2021). To study embryonic development, stem cell differentiation, and reproductive biotechnology, it is necessary to maintain ESCs in an in vitro environment for an extended period of time. Production of autophagy maintains the homeostasis of stem cells as it removes damaged cells, decreases the production of reactive oxygen species (ROS), regulates metabolism, maintains mitochondrial contents, and prevents immature differentiation of stem cells (Chen *et al.,* 2018; Ames *et al.,* 2023; Zhao *et al.,* 2025). To date,

the growth and maintenance of chicken ESCs is still challenging, and several factors have been discovered that are responsible for decreased efficiency.

Rapamycin belongs to a class of macrolides and is proven to be a promoter of autophagy. Several studies have determined its anti-cancer and anti-aging roles (Ganesh and Subathra Devi, 2023; Blagosklonny, 2023). However, its role in maintenance of chicken ESCs has not been studied yet. Rapamycin is a well-known mTOR inhibitor and has been shown to maintain the physiological numbers and self-renewal of stem cells (Chen *et al.,* 2011; Sheppard *et al.,* 2024). In human mesenchymal stem cells (MSCs), the blocking of mTOR target pathways prevented the development of age-related phenotypes via maintenance of normal MSC morphology. These cells showed higher proliferation and clonogenic frequency after several passages (Gharibi *et al.,* 2014). Similarly, rat MSCs pretreated with rapamycin resulted in increased autophagy and lysosome numbers, which improved the survival and maintenance of transplanted cells in the case of myocardial infarction. This increased survival of transplanted cells was linked to higher levels of IGF1, SDF1, HGF, and VEGF. At the same time, the expression of TNF-α and IL-1β was downregulated in rapamycin pre-treated transplanted cells, which determines the role of rapamycin in survival and maintenance of stem cells (Li *et al.,* 2020). Additionally, an increase in the expression of genes related to autophagy was observed in human induced pluripotent cells (IPSCs) after treatment with rapamycin. These genes, LC3B, ATG5, ATG3, ATG12, ATG101, ATG13, BECLIN-1, AMPKα, and ULK1/2, showed increased expression along with decreased expression of the NANOG gene. These molecular changes induced the formation of spontaneous embryoid bodies (EB) and subsequent differentiation into 3 germ layers (Sotthibundhu *et al.,* 2016).

Several studies on cancer cells have demonstrated the role of rapamycin as a tumor suppressor. Rapamycin treatment resulted in the suppression of breast cancer, colorectal cancer, urothelial cancer, prostate cancer, lung cancer, and different kinds of meningiomas (Hansel *et al.,* 2010; Imrali *et al.,* 2016; Pinker and Barciszewska, 2022; Blagosklonny, 2023; Rodero *et al.,* 2025). Rapamycin and its derivatives mainly suppress these types of tumors via blocking mTOR/Akt/PI3K pathways. Higher levels of ROS have been linked with mTOR signaling pathway, which reduces hematopoietic stem cells and their self-renewal ability (Jang and Sharkis, 2007). Moreover, osteogenic differentiation of MSC was activated after the inhibition of mTOR/Akt signaling, which might be due to the decreased ROS generation and upregulation of pluripotency genes (Gharibi *et al.,* 2014). Histone acetyl-transferase 1 (HAT1) directly regulates oxidative stress and the production of ROS species, as shown in HAT1 knockdown mice (Nagarajan *et al.,* 2019). Both rapamycin and HAT1 play crucial roles in the regulation of oxidative stress and cellular metabolism (Agudelo Garcia *et al.,* 2020; Lee *et al.,* 2024; Rossin *et al.,* 2025; Dou *et al.,* 2026). The embryonic stem cell culture requires a threshold level of ROS and ongoing autophagy to get rid of damaged cells. The role of rapamycin on the expression of tumor, metabolism, and ROS-related proteins in chicken ESCs has not been determined yet. In the current study, we determined the differential expression of proteins in rapamycin-treated chicken ESCs.

MATERIALS AND METHODS

Ethic statement and experimental procedure: All the experiments involving the use of animals were approved by the Institutional Animal Care Committee Yangzhou University (permit number: SYXK (Su) IACUC 2012-0029 dated 09-12-2012). All the experiments were conducted in strict accordance with the Administration of Affairs Concerning Experimental Animals regulations and were approved by the State Council of the People's Republic of China. The outline of the experimental procedures conducted during the present study is elaborated in Fig. 1A.

ESC collection, culture and treatment: Twenty fertilized one-day-old eggs were procured from the Poultry Research Institute of Chinese Academy of Agricultural Science, Yangzhou, China. ESCs were collected and purified from the blastoderm region after culture for 24h at 37°C and 5% CO_2 following our lab protocols (Zhang *et al.,* 2013). Briefly, ESCs were collected by cracking the wider end of the egg with sterilized forceps. The inner membrane was removed to expose the blastoderm, which was precisely cut to extract the embryos using fine scissors. These embryos were transferred to tissue culture petri dishes and washed with PBS to remove extra egg yolk. Magnesium and calcium-free phosphate-buffered saline (PBS) was used to rinse the cells and remove the egg yolk. These cells were transferred and incubated for digestion in new petri dishes containing PBS, trypsin (0.25%), and EDTA (0.04%) after washing with PBS to obtain a single cell type, which was identified as described in the next section. After digestion, cells were centrifuged for 10min at 1000rpm. Supernatants were removed, and the sedimented ESCs were collected in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, N.Y., USA) which was supplemented with non-essential amino acids (0.4%), penicillin (0.5%), FBS (10%), and chicken serum (2%). The cells were randomly allocated into 6 Petri plates, 3 for rapamycin treatment and 3 for the control group (untreated). Rapamycin 0.5µm (Cat. No. HY-10219, MCE, Monmouth, JN, USA) was used to treat the ESCs, and the culture medium was replaced every 48h depending on the growth and confluency of ESCs.

Identification of ESCs: Before treatment of ESCs, cell morphology was observed under an inverted microscope to identify the cells. Additionally, immunocytochemistry staining and alkaline phosphatase (ALP) activity were performed to identify the chicken ESCs as described previously (Zhang *et al.,* 2013). After culture, ESCs were rinsed twice with pre-warmed, sterile basal DMEM/F-12 medium. The alkaline phosphatase (AP) working solution was prepared by immediately diluting the 0.1 mM (500×) LIVE AP Stain stock solution (Cat. No. A14353, Life Technologies) 1:500 in basal DMEM/F-12 medium. ESCs were treated with AP working solution for 30min and incubated at 37°C in the

dark. ESCs were washed twice with basal medium, and then fresh basal medium was added to visualize the cells using a FITC filter. To identify the pluripotency markers (SOX2 and SSEA1) of ESCs, paraformaldehyde (4%, Solarbio, P1110) was used, and cells were fixed and left for 30min at room temperature. To remove paraformaldehyde, cells were washed three times with phosphate-buffered saline (PBS, Solarbio, P1020). The ESCs were permeabilized and blocked with a solution containing 1.5% BSA (Solarbio, A8021) and 0.1% Triton X-100 (Solarbio, T8200) for 40min at room temperature, after which primary antibodies against SOX2 (Sigma, AB5603, 1:100 dilution) and SSEA1 (DSHB, MC480, 1:100) were added and cells were incubated at 4°C for 12h. Subsequently, the cells were incubated with Alexa Fluor 555-conjugated anti-rabbit IgG (H+L, Abcam, ab150078, 1:1000) and Alexa Fluor 488-conjugated anti-mouse IgG (H+L, Abcam, ab150113, 1:1000) antibodies for 1h at room temperature. After incubation and washing, the cells were washed with PBS and counterstained with DAPI (Beyotime, P0131) for 5min at room temperature and finally observed under Leica DMi8 immunofluorescence microscope (Leica Microsystems, Wetzler, Germany) using LAS X software (version 3.5.7, Leica Microsystems). 20x magnification was used to acquire all the images with identical exposure using excitation and emission wavelengths of 358 and 461nm, respectively.

Protein extraction, quantification and SDS-page electrophoresis: After culture of ESCs the cells from all 6 Petri dishes (3-ESC-Rapa and 3-ESC-Blank) were frozen at -80°C before further analysis and then transferred to 1.5mL Eppendorf tubes and treated with RIPA lysis buffer (Beyotime Biotechnology, B0013B, Nantong, China), which was further lysed with sonication. Samples were centrifuged at 15,000 rpm for 15min, and the insoluble particles were removed. The protein concentration was measured by a BCA assay kit (AR0146, Boster Biotechnology Co., Ltd., Pleasanton, CA, USA) according to the manufacturer's protocol.

10μg proteins from each sample were loaded into SDS-PAGE gel and separated according to their molecular weight. The gel was separated from the glass apparatus and transferred to a dish containing Milli-Q water. The Milli-Q water was used to wash the gels 3 times for 10min on a shaker, and the water was removed completely. Subsequently, the gel was stained with Coomassie Brilliant Blue (CBB) stain and incubated overnight. After incubation, the stain was removed, and the gel was washed with Milli-Q water until the clear bands were visible. The gel was scanned by an automatic digital gel image analysis system (Tanon 1600R, Shanghai Tanon Life Science Co., Ltd, Shanghai, China).

Enzymatic hydrolysis, TMT labeling and desalination: 100μg of protein was mixed with reducing agent (8M Urea, 10mM DTT, 100mM TEAB having pH 8.0) and placed in a filtration tube. The mixture was incubated for 1h at 60°C and then added with indole-3-acetic acid (IAA) to obtain a 50 mM concentration. The supernatant was removed after centrifugation (12000rpm) for 20min at 4°C. 300mM TEAB was added, and the mixture was again centrifuged (12000 rpm) for 20min at 4°C. After discarding the supernatant, 300mM TEAB (100 μL) along with 3μL of trypsin (1μg/μL) was added. The solution was incubated (for 12h) for digestion at 37°C. These peptides were centrifuged at 12000rpm for 20 min, and the supernatant was discarded; the final 50μL of 200mM TEAB was added and centrifuged again at 12000rpm for 20min and lyophilized.

For TMT labeling, the lyophilized samples were resuspended in 200mM TEAB (100μL) and 40μL of each sample was transferred to tubes and labelled. Aceto-nitrile (80μL) was added to the TMT reagent and mixed. 41μL of this TMT and acetonitrile mixture was added to each sample and left for 1h after thorough mixing. The labelled peptides were lyophilized and stored at -80°C.

To adjust the pH and desalt the samples 2-4μL of H3PO4 was added and subjected to a C18 reverse-phase SPE column, respectively. Initially, the column was washed twice with 1mL methanol, followed by 3 times washing with TFA/H2O (0.1%). Then the samples were loaded on the column, and the peptides were eluted with 90% CAN/H2O containing 0.1% TFA and lyophilized.

Mass spectrometry analysis: Before mass spectrometry, all the samples were mixed with an iRT kit (Biognosys, ThermoFisher) which contains 11 synthesized stable amino acids for quality control and calibration. These amino acids do not exist in nature and do not affect the samples to be tested. All the experiments were performed in triplicate using Triple TOF 5600 (SCIEX, USA) mass spectrometer equipped with Nanospray III source (SCIEX, USA). An Eksignet nanoLC-1D plus system (SCIEX, USA), which was equipped with C18 capillary trap column (3cm × 100μM) was used to load the samples, and a C18 column (15cm × 75μM) was used to separate the samples. The system settings were as follows: flow rate 300nL/min with linear gradients: 0~0.5min and 95%~92% A; 0.5~48min and 92%~74% A; 48~61min and 74%~62% A; 61~61.1min and 62%~15% A; 61.1~67min and 15% A; 67~67; 1min and 15%~95% A; 67.1~70min and 95% A. Mobile phase A was equal to 2% ACN/0.1% FA and B was equal to 95% ACN/0.1% FA. The data were generated by 2.4kV ion spray, 35psi curtain gas, 5psi nebulizer gas, and 150°C interface heater temperature. The mass spectrometry scan time for the 400-1500 mass range was 250ms and for IDA, 30 MS/MS and 100-1500 mass range was 80ms. The mass spectrometer peaks of more than 260 intensity with charge ranging between 2 and 5 were acquired. For CID fragmentation for MS/MS spectra, a rolling collision energy voltage was used. Proteins with expression values of ≥50% were accounted for, and missing values were filled with mean values of the same groups. Median normalization and log-2-log-transformation were used to acquire trusted proteins.

Database search and statistical analysis: ProteinPilot (v. 5.0) software was used to search all the obtained raw data and compared with protein data available online. Database search was performed with specificity of

cysteine alkylation and trypsin digestion. To quantify the proteins and peptides, the TMT labeling method was selected, and a global false discovery rate (FDR) of < 1 % was used for at least 2 peptides to acquire the results. The P-value was calculated using Student's *t-test,* and P<0.05 was considered significant. Furthermore, log2FC was set as standard for differentiating the proteins in both ESC-Rapa and ESC-Blank groups.

RESULTS

Identification of chicken embryonic stem cells: ESCs express higher levels of pluripotency markers such as SOX2 and SSEA1. These cells demonstrated higher activity of alkaline phosphatase, which is a direct measure of pluripotency. In this study, before conducting extensive proteomic analysis, the success of ESC collection was determined through the expression of SOX2 and SSEA1 antibodies along with the activity of alkaline phosphatase (Fig. 1B and 1C).

Expression of proteins in chicken embryonic stem cells: A total of 4495 proteins were expressed in both untreated and rapamycin-treated embryonic stem cells. Out of which 4044 proteins showed non-significant (P>0.05) difference in their expression between both ESC-Blank and ESC-Rapa groups. However, 126 proteins were found to be significantly (P<0.05) upregulated, and 325 proteins were found to be significantly (P<0.05) downregulated (Fig. 2A). Principal component analysis (PCA) was carried out to summarize the differences between two groups based on the most variable components which showed 33.56% and 25.65% variability for principal component-1 (PC-1) and principal component-2 (PC2), respectively. Moreover, PCA showed that ESC-Blank and ESC-Rapa treated groups are distinct from each other based on their protein expressions (Fig. 2B). Next, the difference between protein expressions of the two groups was elaborated through hierarchical cluster analysis and is presented along with a heat map based on Fold change (FC) as shown in the figure. A total of 451 differentially expressed proteins are shown, which showed visible differences between ESC-Blank and ESC-Rapa groups (Fig. 2C).

Correlation between differentially expressed proteins: To further elaborate the relationship between the differentially expressed proteins with cellular components, the top 60 differentially expressed proteins were selected and visualized in the form of a chord plot (Fig. 3A). This visualization showed that most of the differentially expressed proteins were related to nucleoplasm of chicken embryonic stem cells followed by Cajal bodies, SWI/SNF complex, and NBAF complex. Some proteins from the top 60 differentially expressed proteins were also found to positively influence the smooth muscles of vessels. Additionally, to visualize the correlation between these proteins, the top 50 differentially expressed proteins were selected and presented in the form of a correlation matrix (Fig. 3B).

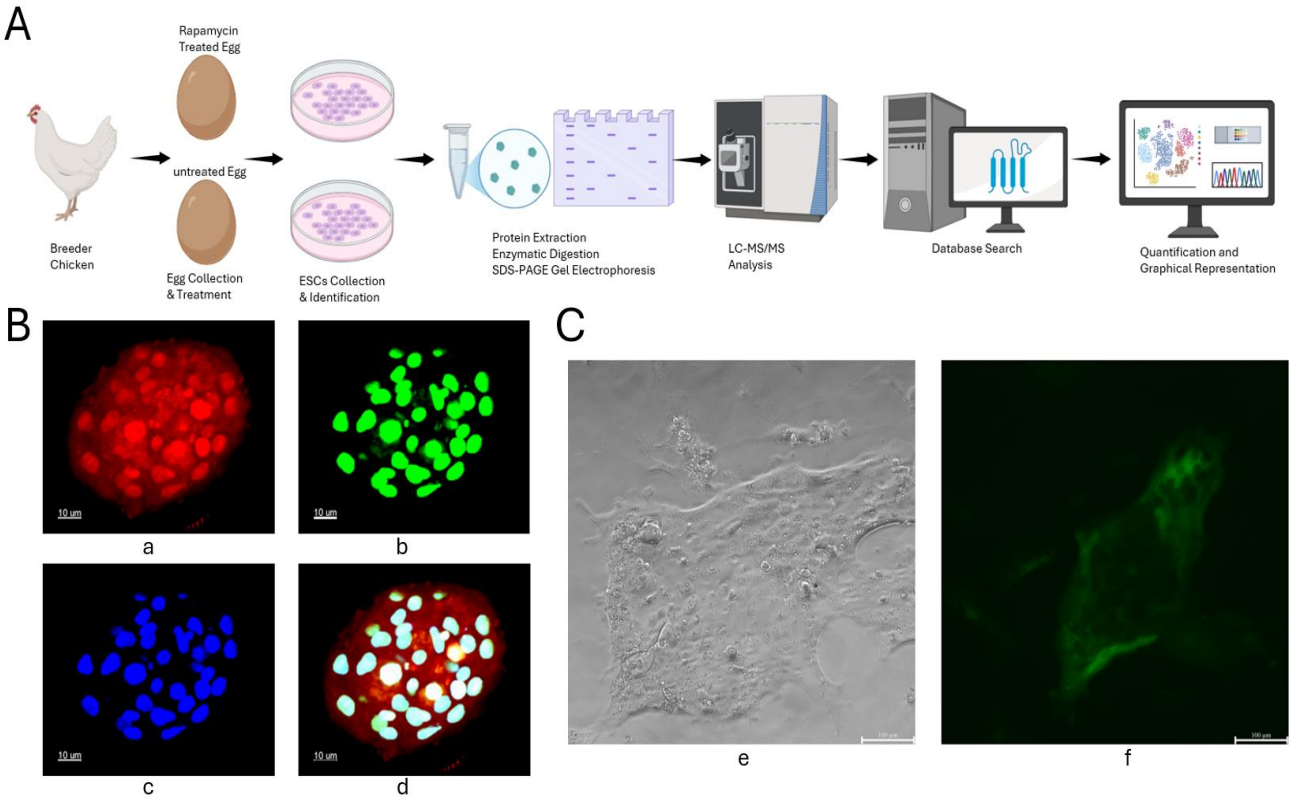

Fig. 1: Experimental design and identification of ESCs. Experimental procedure from fertilized egg collection to proteomic analysis after treatment with rapamycin (A); Identification of ESCs through immunofluorescence and expression of SOX2 and SSEA1 pluripotency markers. a, b, c and d represent the SOX2, SSEA1, DAPI and merge diagrams, respectively, during the identification process (B); Alkaline phosphatase activity indicating the pluripotency of cells. e and f represent the bright field and immunofluorescence images during alkaline phosphatase activity detection (scale bar 100µm) (C).

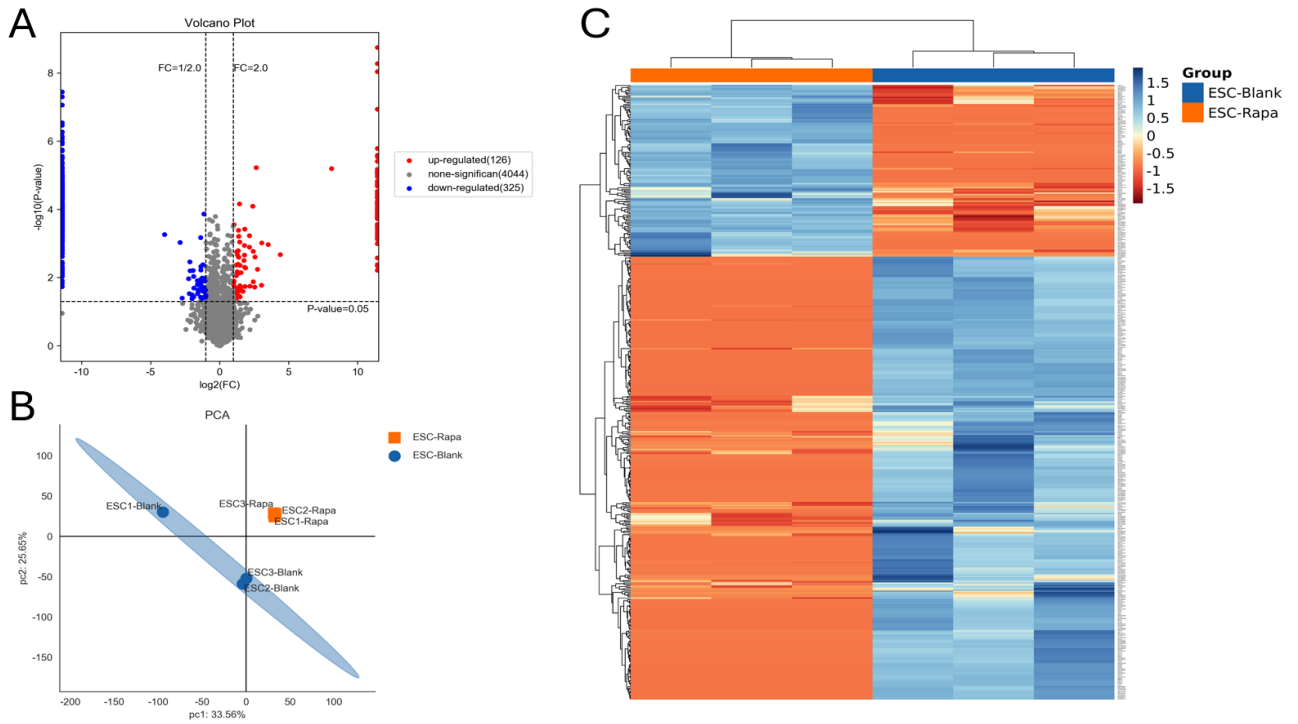

Fig. 2: Rapamycin treatment resulted in differential expression of proteins as compared to untreated ESCs of chicken. Up- and down-regulated proteins in rapamycin-treated vs untreated ESCs (A); principal component analysis indicating correlations among treatment groups (B); heat map and hierarchical clustering indicating the degree of differential protein expression. Red indicates downregulated proteins, whereas blue indicates upregulated proteins (C).

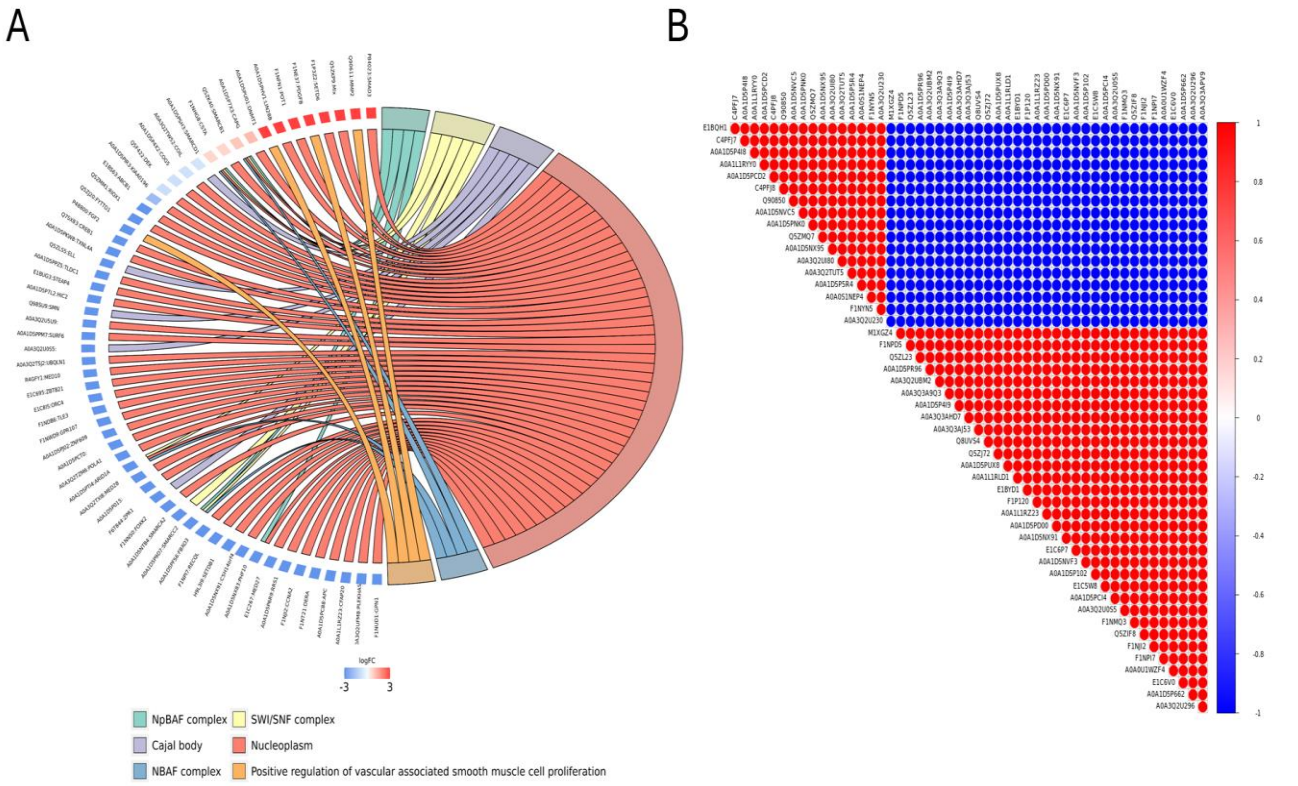

Fig. 3: Correlation among differentially expressed proteins. Chord plot indicating the correlation among top 60 differentially expressed proteins. The intensity of orange or red color, indicate a strong negative or positive correlation, respectively (A); Top 50 differentially expressed proteins presented in the form of correlation matrix. Intensity of red or blue indicate strong positive or strong negative correlations respectively (B).

Gene ontology (go) analysis: GO analysis was carried out to determine the biological processes (BP), cellular component (CC), and molecular functions (MF) governed by these differentially expressed proteins. The higher percentage of top upregulated proteins was linked to cellular components, as shown in Fig. 4A. The prominent cellular functions were related to extracellular space, extracellular region, and lysosomal membrane. A

few proteins were found to be related to egg yolk, sarcomere, and SEI/SNF complex. However, top downregulated differentially expressed proteins were involved in glucose metabolism and toll-like receptor signaling pathway-related biological processes (Fig. 4B). Moreover, the cellular components were related to cytosol, nucleoplasm, nuclear body, and Cajal body of chicken ESCs. The important molecular functions governed by these proteins were related to protein binding, as shown in the figure.

KEGG pathway analysis and protein-protein interactions: To predict the functions of differentially expressed proteins in cellular processes, metabolism, genetic regulations, and signaling pathways, Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for up and down-regulated proteins. Most of the upregulated proteins were involved in the pathways related to metabolism and mTOR signaling (Fig. 5A). The important metabolic pathways were lysine degradation, phenylalanine metabolism, and glutathione metabolism. Organismal system-related pathways were enriched in progesterone-mediated oocyte maturation and GnRH signaling-related pathways. Additionally, the top downregulated pathways were enriched in autophagy, notch signaling, DNA replication, lysine degradation, fatty acid biosynthesis and pyrimidine metabolism (Fig. 5B). To specify the number of up and downregulated proteins in specific KEGG pathway further analysis was performed as presented in figure below (Fig. 5C). A total of 5 up and 5 downregulated proteins were identified in cell growth and death related pathways. Similarly, in the carbohydrate metabolic pathway, 3 proteins were upregulated, and 5 proteins were downregulated. Proteins related to other metabolic pathways are mentioned in the figure below. These proteins were further elucidated to determine the interactions between up- and down-regulated proteins in ESC-Blank and ESC-Rapa groups (Fig. 5D). Several proteins related to metabolism, tumors, cell signaling, growth, and degradation were identified to interact with each other.

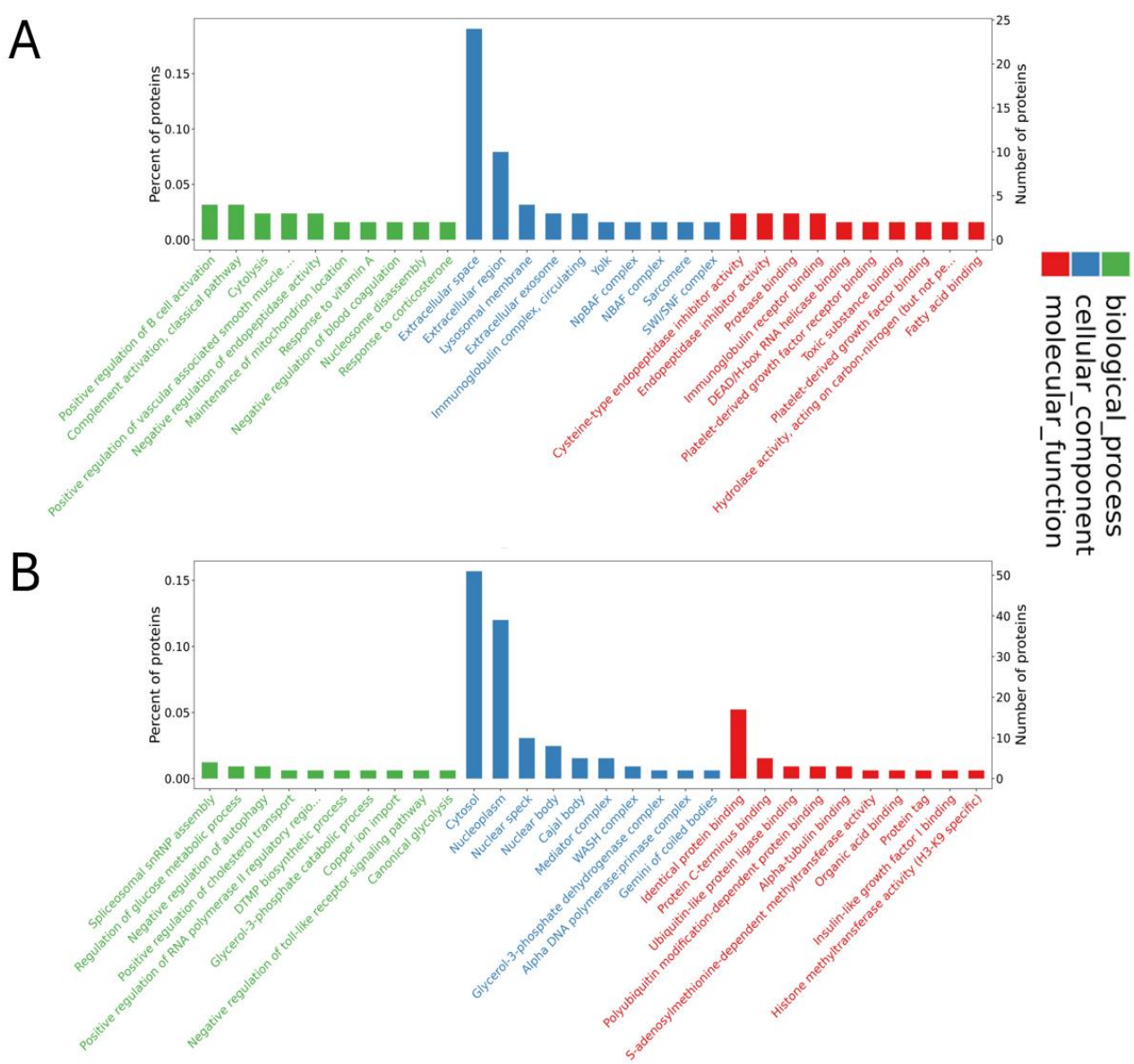

Fig. 4: GO analysis of top differentially expressed proteins involved BP, CC and MF. Top upregulated differentially expressed proteins (A); Top downregulated differentially expressed proteins (B).

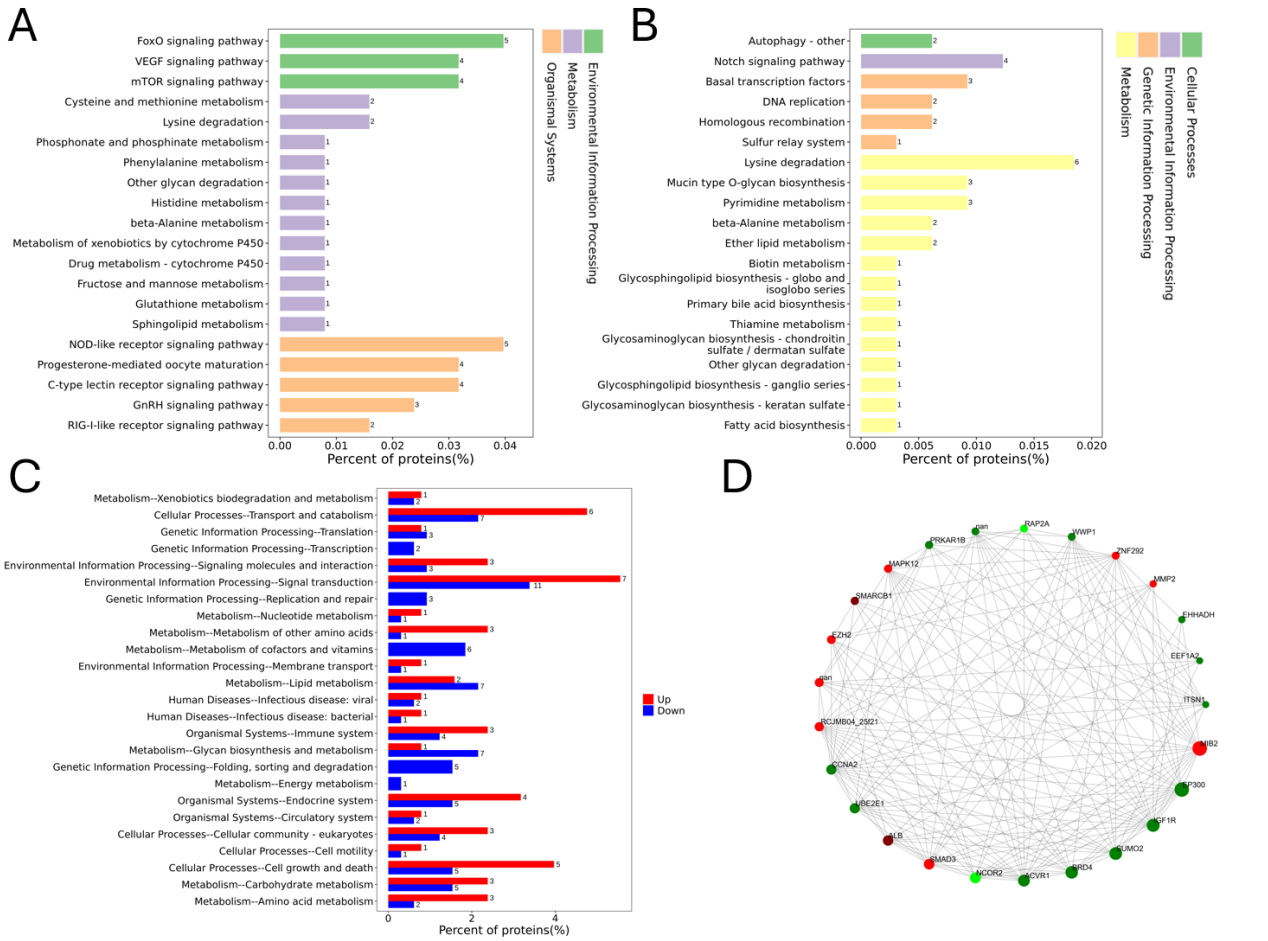

Fig. 5: KEGG pathway analysis and protein-protein interaction among top differentially expressed proteins between rapamycin treated and untreated chicken ESCs. KEGG pathway analysis of top upregulated proteins (A); KEGG pathway analysis of top downregulated proteins (B); Classification of differentially expressed proteins in different pathways (C); protein-protein interactions among differentially expressed proteins (D).

DISCUSSION

In vitro culture of chicken embryonic stem cells requires specific culture media and factors for their maintenance and proliferation. Many studies have reported the successful culture of these pluripotent stem cells in Dulbecco's modified Eagle's medium (DMEN); however, unlike other mammals and non-mammals, chicken embryonic stem cells are prone to osmotic stress posed by this medium (van de Lavoir and Mather-Love, 2006). In routine, the DMEM is supplemented with fetal bovine serum (FBS) to culture mouse ESCs, as this provides growth factors for cell maintenance. However, the FBS employed alone for the culture of mouse ESCs is not suitable for culturing chicken ESCs. In addition to FBS, chicken ESCs require chicken serum for their growth and long-term maintenance in vitro (Horiuchi *et al.* 2006). The propagation of chicken ESCs and other stem cells provides an ideal opportunity to generate transgenic animals to produce human and animal recombinant proteins and vaccines (Han *et al.,* 2015). However, long-term maintenance of chicken embryonic stem cells is still a challenge, and regulation of genes and proteins could improve the propagation of chicken ESCs.

The data obtained after mass spectrometry were analyzed and showed lower expression of cancer-related proteins in ESCs-Rapa group. The prominent oncogenic proteins which were downregulated are HDAC2, YWHAG, PRDX4, HMGB3, SMARCB1 and VDAC2. Several human-based studies have shown that these oncogenic genes were upregulated during breast cancer, prostate cancer, colorectal cancer, urothelial cancer and meningiomas. In a human breast cancer study, the involvement of HDAC2 and its overexpression was linked with the clinicopathological state of cancer, which resulted in poor prognosis (Shan *et al.,* 2017). Similarly, the role of YWHAG in the progression of colorectal cancer, pancreatic cancer, bladder cancer and adenocarcinoma of the lungs has been shown in many studies (Xu *et al.,* 2021, Wang *et al.,* 2024, Tian *et al.,* 2024, Zheng *et al.,* 2025). During these studies, the pathways that were involved in the progression of cancer were CTTN-Wnt/β catenin, ERK/MAPK and JAK2/STAT3. Studies on chicken ESCs and other stem cells showed that blockade of Wnt/β catenin pathway is necessary to maintain the pluripotency of stem cells, as upregulation of this pathway led to differentiation of ESCs into spermatogonial stem cells (He *et al.,* 2018, Kajihara *et al.,* 2024,*et al.,*). In our study, the treatment of chicken ESCs with rapamycin decreased the expression of YWHAG, which might lead to downregulation of Wnt/b-catenin pathway and maintain the pluripotency potential of chicken ESCs. The upregulation of these proteins might result in unwanted proliferation of chicken ESCs with damaged DNA and mitochondria. This could be explained by the fact that proliferating ESCs undergo extensive

autophagy, and increased expression of genes related to autophagy has been observed in many studies.

The production of ROS during in vitro culture of stem cells decreases the viability and proliferation of these cells. A study on chicken embryo fibroblast cells has demonstrated the effect of ROS on in vitro culture and proliferation of cells. Increased production of ROS was directly related to increased expression of mTOR protein, which severely enhanced autophagy and decreased the viability of fibroblast cells (Shen *et al.,* 2022). Mesenchymal stem cells retain their ability to regenerate and differentiate into osteogenic cells due to expression of autophagy related genes. This autophagy regulates production of ROS and expression of p53 as demonstrated in bone marrow mesenchymal stem cells (Ma *et al.,* 2018). These studies indicate that both autophagy and ROS affect each other during the culture and proliferation of stem cells. Similarly, in our study, we have observed a decrease in oxidative stress-related proteins, i.e., HSDL1, ACOX1, GPX1 and HSP25 in rapamycin-treated embryonic stem cells. This could be related to controlled levels of autophagy in chicken ESCs after treatment with rapamycin, as rapamycin removes the damaged cells that contribute to excessive ROS production. However, the exact mechanism through which rapamycin reduced the expression of these proteins needs further investigation.

The chicken embryonic stem cells rely mostly on glycolysis for metabolism and energy production instead of oxidative phosphorylation (OXPHOS) and the tricarboxylic acid cycle (TCA). Several studies have reported that the mitochondria of stem cells are immature and lack cristae, unlike those of somatic or differentiated stem cells. In our study, we have seen no difference in the expression of metabolic proteins related to mitochondria (IDH2, MDH2, and ACO1) in chicken ESCs after treatment with rapamycin. However, an increase in cytosolic enzymes related to glucose and glycogen metabolism (VNN1 and G6PC3) was observed in rapamycin-treated chicken ESCs. The current study mainly focused on the differential expression of proteins in rapamycin-treated and untreated chicken ESCs, which further requires validation through apoptosis, proliferation, and ROS quantification assays. Furthermore, the differentially expressed proteins have not been validated at RNA and protein levels through qRT-PCR and western blotting, which represents a limitation of the current study. However, this unbiased proteomic approach serves as a hypothesis-generating resource for future mechanistic studies.

Conclusions: Rapamycin is a potent cancer inhibitor as shown by several previous studies. In the present study, treatment of chicken embryonic stem cells (ESCs) with rapamycin resulted in the downregulation of several cancer-related proteins, including HDAC2, YWHAG, PRDX4, HMGB3, SMARCB1 and VDAC2, which may suggest a potent role of rapamycin in suppression of cancer. Moreover, rapamycin resulted in the downregulation of oxidative stress-related proteins including HSDL1, ACOX1, GPX1 and HSP25, suggesting its role in the regulation of oxidation-reduction in chicken stem cells. This study provides a foundation for future research on the molecular mechanisms through which rapamycin suppresses oncogenic and oxidative stress-related genes and proteins. Additionally, it will enhance the understanding and potential applications of these mechanisms to study stem cell pluripotency, differentiation and developmental biology.

Authors Contribution: conceptualization and writing; M.A.A., reviewing; S.U., K.J, editing; J.S., H.S., and K.J., data curation; M.A.A, M.K.N., and U.A., review and editing; M.A.A., K.J., and B.L., supervision; K.J and B.L., funding acquisition; B.L.

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

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