# Chicken telomerase reverse transcriptase mediates LMH cell pyroptosis by regulating the nuclear factor-kappa B signaling pathway

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**ABSTRACT** The activation of human telomerase reverse transcriptase is regulated by the nuclear factor kappa B (**NF-κB**) signaling pathway to various degrees to promote the occurrence and development of tumors. However, the regulatory roles of chicken telomerase reverse transcriptase (**chTERT**) and the NF-κB signaling pathway in chickens are still elusive, particularly in respect to the regulation of cell pyroptosis. In this study, we found that chTERT upregulated the expression of p65 and p50, downregulated the expression of IκBα, promoted the phosphorylation of p65, p50, and IκBα, and significantly increased the transcript levels of the inflammatory cytokines IFNγ, TNFα, and IL-6 in LMH cells. The activity of NF-κB was significantly decreased after siRNA-mediated chTERT silencing. The expression of chTERT and telomerase activity were also significantly decreased when the NF-κB signaling pathway was blocked by p65 siRNA, MG132 or BAY 11-7082. In cells treated with LPS, the activity of NF-κB signaling pathway and the expression of chTERT were significantly upregulated. All of the results suggested that chTERT and the NF-κB pathway could regulate each other, reciprocally. Moreover, the expression of Caspase-1, NLRP3, GSDMA, IL-18, and IL-1β and caused membrane perforation, suggesting the development of pyroptosis by chTERT in LMH cells. And the expression of caspase-11 did not significantly increased in chTERT overexpression group. Genetic silence of NF-κB p65 or chTERT gene by siRNA suppressed the expression of these proinflammatory cytokines, indicating that chTERT mediates pyroptosis by regulating the NF-κB signaling pathway in LMH cells.

**Key words:** chicken telomerase reverse transcriptase, NF-κB signaling pathway, cell pyroptosis, LMH cells

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## INTRODUCTION

Telomeres are small pieces of the DNA-protein complex at the end of linear chromosomes of eukaryotic cells and are composed of a highly conserved (TTAGGG)n repeat sequence and telomere-binding protein (Young et al., 2021). In normal cells, cell cycle arrest, senescence or apoptosis occur after continuous cell division. The shortening of telomeres is one of the important factors associated with cell death and the prevention of infinite division (Samad et al., 2021). Telomerase is a nuclear protein reverse transcriptase that is responsible for elongating telomeres in cells and is composed of human telomerase reverse transcriptase (**hTERT**), the telomerase RNA component and the telomerase associated protein (Ghareghomi et al., 2021). Telomerase uses telomerase RNA as a template to synthesize telomere DNA under the catalysis of hTERT and ligate this DNA to the ends of chromosomes, thereby maintaining the stability of telomere length and inducing unlimited cell division and proliferation (Bernabe-Garcia et al., 2021). Studies have shown that hTERT gene expression is the core determinant of telomerase activity and that the hTERT gene is overexpressed in most malignant human tumors. Telomerase activation is considered to be a key step in carcinogenesis and is one of the most common molecular markers in a wide range of malignant tumors (McKelvey et al., 2020; Roggisch et al., 2020; Wang and



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Sun, 2020). Current research progress showed that chicken telomere is the longest known telomere, and their DNA accounts for at least 3% to 4% of genomic DNA, which is approximately 10 times higher than the amount of DNA found in the human genome (Delany et al., 2003). The biological function of chicken telomerase reverse transcriptase (**chTERT**) is similar to that of the human variant, and it is also the core component that regulates chicken telomerase (Wang et al., 2017). However, there have been few reports on chTERT-mediated regulation of cell growth and development, as well as the occurrence and development of tumors, and further exploration is needed.

There are several signaling pathways involved in the transcriptional regulation of hTERT, among which the nuclear factor kappa B (**NF-κB**) signaling pathway is one of the most commonly involved and plays an important role in the transcriptional regulation of hTERT (Pestana et al., 2017). For example, the hTERT protein can directly interact with p65 in human IgA-producing myeloma cells (Akiyama et al., 2003). NF-κB p65 modulates hTERT in the HepG2 hepatoma cell line (Zuo et al., 2011). When hTERT was overexpressed in human skin fibroblasts, p-NF-κB p65 was activated and translocated to the nucleus, and hTERT promoted autophagy by activating NF-κB (Liu et al., 2016). It was also found that hTERT cooperated with p65 to increase the transcription level of NF-κB and continued to drive cell proliferation, resist apoptosis and produce a chronic inflammatory state (Ghosh et al., 2012). In atherosclerotic plaques, miR-216a regulated the downstream NF-κB pathway to promote macrophage telomerase activation and hTERT expression (Yang et al., 2019). These results suggested that hTERT is a potential target of NF-κB (Wu et al., 2017). However, in avian tumor diseases such as Marek's disease and avian leukosis, the regulatory mechanism of chTERT and the NF-κB signaling pathway is still unclear. Therefore, it is necessary to explore the relationship between chTERT and the NF-κB signaling pathway, which may provide a theoretical basis for in-depth study of the role of chTERT in avian tumor diseases.

Pyroptosis is a type of programmed cell death triggered by cysteinyl aspartate specific proteinase (Caspase), which mainly mediates the activation of various caspase through inflammasomes (Chen et al., 2021). Caspase activates and cleaves gasdermin, resulting in cell membrane perforation, accompanied by the release of a large number of inflammatory factors (Yin et al., 2021). The NF-κB signaling pathway has been regarded as a key pathway that regulates the inflammatory response (Allaeys et al., 2021). The activation of NF-κB is the first signal of nucleotide-binding oligomerization domain-like receptor 3 (**NLRP3**) inflammasome activation, and activation of the NLRP3 inflammasome and its effectors Caspase-1 and IL-1β is regulated by NF-κB (Lee et al., 2021). Studies have confirmed that respiratory syncytial virus infection can induce NF-κB activation and activate the gene expression of NLRP3 (Segovia et al., 2012). In addition, inhibiting the NF-κB signaling pathway can downregulate the expression level of NLRP3 (Zhao et al., 2021). Furthermore, blocking NF-κB signaling can downregulate the mRNA levels of the inflammatory cytokines IL-1β, IL-6, TNF-α, and IL-18, as well as the levels of the pyroptosis-related proteins NLRP3, Caspase-1 and gasdermin D (**GSDMD**) (Shao et al., 2020). After silencing p65 or NLRP3, the expression of Caspase-1, IL-1β, and IL-18 decreased, which attenuated pyroptosis in a mouse macrophage cell line. These studies show that NF-κB is a key factor in regulating pyroptosis (Xu et al., 2021a). It has also been reported that when broilers were infected with *Salmonella* typhimurium, the transcription levels of gasdermin A (**GSDMA**) were significantly increased, which were involved in the occurrence and development of inflammation. Moreover, there have been few reports on the effects of hTERT or chTERT on pyroptosis. According to these studies, the NF-κB signaling pathway may be involved in the occurrence of pyroptosis by regulating inflammatory cytokines, but further confirmation is needed. However, the relationship among and regulatory mechanism of chTERT, the NF-κB signaling pathway, and pyroptosis are currently unclear. Therefore, this study aimed to explore the interaction between chTERT and NF-κB signaling pathways in LMH cells and its regulatory mechanism in pyroptosis.

## MATERIALS AND MTEHODS

### Antibodies

Anti-NF-κB p65 (Cat. AF5006), anti-phospho-NF-κB p65 (Cat. AF2006), anti-IκBα (Cat. AF5002), and anti-phospho-IκBα (Cat. AF2002) primary antibodies were purchased from Affinity Biosciences Ltd., Pottstown, PA. Anti-NF-κB p50 (Cat. sc-166588) and anti-phospho-NF-κB p50 (Cat. sc-271908) primary antibodies were purchased from Santa Cruz Biotechnology, Dallas, TX. Anti-GAPDH (Cat. ab181602) primary antibodies were purchased from Abcam, Inc., Cambridge, UK. Anti-HA tag (Cat. 26183) was purchased from Invitrogen, Thermo Fisher Scientific, Inc., Carlsbad, CA. Goat anti-rabbit IgG (Cat. 926-32211) and goat anti-mouse IgG (Cat. 926-32210) secondary antibodies were purchased from LI-COR Biosciences Ltd., Lincoln, NE. The LMH cell line and 293T cell line were provided by Professor Ming Liao of South China Agricultural University.

### Cell Culture

Avian leghorn male hepatoma (**LMH**) cell line stably overexpressing the chTERT (HA-tag) gene (LMH-chTERT) and control cells (LMH-NC) were constructed by our laboratory in previous study (Xiang et al., 2021). The cells were cultured in DMEM/F12 (Gibco, Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum and maintained at 37°C and 5% $CO_2$. Before inoculating the resuspended cells into the culture dish, the cell culture plate should be coated with Poly-

D-lysine hydrobromide (Beyotime, Shanghai, China) for about 5 min to enhance the adherence of the cells.

## Cell Treatment

To inhibit the expression of NF-κB and chTERT, cells were transfected with p65-specific small interfering RNA (siRNA) (si-p65, 5′- GCAUUGAGGAGAAGCGCAATT-3′, 5′- UUGCGCUUCUCCUCAAUGCTT-3') and chTERT-specific siRNA (si-chTERT, 5′- GCAUGGAACCUCCUGGCAUTT-3′, 5′-AUGCCAGGAGGUUCCAUGCTT-3') or nonspecific siRNA (si-NC, 5′-ACGUGACACGUUCGGAGAATT-3', 5′- UUCUCCGAA CGUGUCACGUTT -3′) (10 μmol/L) that was synthesized by Shanghai GenePharma Company. Cells were plated in 12-well plates (100,000 cells/well) or 6-well plates (200,000 cells/well) and transfected with siRNA in the presence of siRNA-Mate Transfection Reagent (GenePharma, Shanghai, China). In addition, inhibitors (MG132 and BAY-117082) (Cat. S1748 and S1523, Beyotime, Shanghai, China) and activators (LPS) (Cat. S1732, Beyotime, Shanghai, China) of NF-κB were used to inhibit or activate the activity of the NF-κB signaling pathway (Zuo et al., 2011; Shen et al., 2019), and these reagents were added directly to the cell culture medium. After at least 24 h of treatment, cell viability and mRNA or protein expression were measured by MTT analysis, RT-PCR and Western blotting, respectively.

## MTT Assay

Cytotoxicity was measured using the MTT assay with an Enhanced Cell Counting Kit-8 (Cat. C0009S, Beyotime, Shanghai, China). The detailed procedure was performed according to manufacturer's instructions. Briefly, LMH cells were seeded on 96-well plates at a density of $1 \times 10^4$ cells/well in 200 μL of medium, incubated for 12 h and then treated with MG132, BAY 11-7082, LPS or siRNA at different concentrations. After 72 h, CCK-8 reagent was added to cells in the wells and incubated at 37°C for 2 h, and then the absorption was measured at 450 nm using a microplate reader (BioTek, VT). Relative absorbance was calculated according to the following formula: cell viability = $(OD_{treated} - OD_{blank})/(OD_{untreated} - OD_{blank})$, where OD = optical density at 450 nm.

## Real-Time Fluorescence Quantitative PCR

Total RNA was extracted from LMH cells by using a Total RNA Kit (Cat. 220011, Fastagen Biotech, Shanghai, China) according to the manufacturer's recommendations. First-strand cDNA was synthesized from 500 ng of total RNA template with random primers using a PrimeScript RT reagent kit (Cat. RR036A, TaKaRa, Japan). Real-time PCR was performed using Hieff qPCR SYBR Green Master Mix (Cat. 11201ES03, YEASEN, Shanghai, China) on a CFX96 Real-time PCR System (Bio-Rad, CA). Expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH. Primer information is showed in Table 1 by referring to relevant literatures (Yang et al., 2007; Wang et al., 2018; Liao et al., 2019; Liu et al., 2019; Luborsky et al., 2020).

**Table 1.** Primers used for real-time fluorescence quantitative PCR.

| Genes (Accession) | Primer sequences (5'-3') | Length (bp) |
|---|---|---|
| chTERT (NM_001031007.1) | F: GTAAGACTAAGCCGTGTTGTTG<br>R: CTCCCGAATACTGAAGAGC | 158 |
| p65 (NM_001396396.1) | F: TCAACGCAGGACCTAAAGACAT<br>R: GCAGATAGCCAAGTTCAGGATG | 162 |
| IκBα (NM_001001472.2) | F: CTTCCAGAACAACCTCAGCCAGAC<br>R: CGCAGCCAGCCTTCAGCAG | 92 |
| IFNγ (NM_205149.2) | F: ACCTTCCTGATGGCGTGAAG<br>R: GCGCTGGATTCTCAAGTCGT | 80 |
| TNFα (NM_204267.2) | F: GCCCTTCCTGTAACCAGATG<br>R: ACACGACAGCCAAGTCAACG | 71 |
| IL-6 (HM179640.1) | F: AAATCCCTCCTCGCCAATCT<br>R: CCCTCACGGTCTTCTCCATAAA | 135 |
| IL-1β (HQ329098.1) | F: CAGCCTCAGCGAAGAGACCTT<br>R: ACTGTGGTGTGCTCAGAATCC | 84 |
| IL-18 (HM854281.1) | F: GCAGTACGGCTTAGAGAAAA<br>R: GTACATTCCACTGCCAGATT | 198 |
| NLRP3 (KF318520.1) | F: AGCTACCACACATCTAGGAT<br>R: GGTGTCCAAATCCTCAATCT | 207 |
| Caspase-1 (XM_015295935.3) | F: TTCCTTCAACACCATCTACG<br>R: GGTGAGCTTCTCTGGTTTTA | 209 |
| Caspase-11 (NW_003763474.1) | F: CCCCCACCATCTCAACAAGT<br>R: GTCCCTGAACAGTTCCCACA | 157 |
| Gasdermin A (NM_001031361.1) | F: CCATAGCGAGCACAGCAAAC<br>R: GATGCTGTGGACAGGAACCA | 234 |
| Gasdermin E (NM 001006361.1) | F: ACTGCTGTTCTGCAGTGGAAA<br>R: AGCCTTTCTGCTCACTTGCT | 214 |
| GAPDH (NM_204305.2) | F: GGAAAGTCATCCCTGAGCTG<br>R: GGTCAACAACAGAGACATTGG | 81 |

## Western Blotting

Protein was extracted from LMH cells using fierce RIPA lysis buffer (Cat. P0013B, Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, equal amounts of the total protein were separated by SDS-polyacrylamide gel electrophoresis (**SDS-PAGE**) (Cat. P0012A, Beyotime, Shanghai, China) and then transferred onto a polyvinylidene difluoride (**PVDF**) membrane. The membranes were immunoblotted with primary antibodies at 4°C overnight, followed by fluorescein isothiocyanate-conjugated secondary antibodies at 37°C for one hour. Finally, the membranes were scanned using an Odyssey Infrared Imaging System (LI-COR).

## Telomerase Activity Assay

Telomerase activity was measured by the telomeric repeat amplification protocol (**TRAP**). The TRAP assay was carried out with the TeloTAGGG Telomerase PCR ELISA kit (Cat. 11854666910, Sigma-Aldrich, St. Louis, MS, USA), which was performed according to manufacturer's instructions.

## Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokines related to pyroptosis, such as chicken-specific Caspase-1 (Cat. ml060932), Caspase-11 (Cat. ml391588), NLRP3 (cat. ml521570), Gasdermin A

(**GSDMA**) (Cat. ml696302), IL-18 (Cat. ml059834) and IL-1$\beta$ (Cat. ml059835), were analyzed according to manufacturer's instructions of ELISA kit (Mlbio, Shanghai, China). Briefly, the cell supernatant was collected and centrifuged for 20 min at 1,000 g. Particulates were removed and assayed immediately; 50 $\mu$L of standard or sample was added to the appropriate wells, and blank wells were set. Subsequently, 100 $\mu$L of enzyme conjugate was added to the standard and sample wells except the blank well, covered with an adhesive strip and incubated for 60 min at 37°C. After 4 washes, 50 $\mu$L of Substrate A and 50 $\mu$L of Substrate B were added to each well. The wells were gently mixed and incubated for 15 min at 37°C. Then, 50 $\mu$L of stop solution was added to each well. Next, the OD value was read at 450 nm using a microtiter plate reader within 15 min. The standard curve was generated by plotting the average $OD_{450nm}$ obtained for each of the six standard concentrations on the vertical (X) axis vs. the corresponding concentration on the horizontal (Y) axis. The standard curve was used to determine the amount in the unknown sample.

### Transmission Electron Microscopy (TEM)

LMH cells were seeded on 6-well cell culture plates, and after 24 h of incubation, the cells were harvested and fixed in 2.5% glutaraldehyde for 24 h at 4°C. Subsequently, the cells were rinsed with buffer, fixed with citric acid and dehydrated in a series of ethanol solutions. Then, the cells were permeabilized with different ratios of ethanol: resin and embedded in pure resin, which was polymerized at 70°C. Stained cells were visualized using a transmission electron microscope (Hitachi, Ltd., Japan) to observe the various structures within the cells.

### Statistical Analysis

All data in this article were shown as means ± standard deviation (means ± SD) for at least 3 replicate experiments. Statistical analysis was conducted using the GraphPad software. Differences between groups were analyzed using the ANOVA followed by the Tukey test. *, **, and *** indicate $P$ values less than 0.05, 0.01 and 0.001, respectively. $P$ values of $<0.05$ was considered statistically significant.

## RESULTS

### Effect of MG132, BAY 117082, LPS, and siRNA on LMH Cell Cytotoxicity

To determine the concentration range in which the inhibitors and siRNA suppressed NF-$\kappa$B p65 or chTERT gene expression with an acceptable cell viability level, the cytotoxicity of NF-$\kappa$B signaling pathway inhibitors (MG132, BAY 11-7082) and the siRNA (si-p65, si-chTERT) were assessed with an MTT assay. The cytotoxicity of an activator of this signaling pathway (**LPS**) was also tested. For functional testing, the cells were incubated with an inhibitor or activator at different concentrations for 24 h, 36 h, and 72 h. Similarly, siRNA was transfected into LMH cells at different concentrations for 24 h, 36 h, and 72 h. Over a wide concentration range, MG132, BAY 11-7082, LPS and siRNA induced decreasing cell viability in response to increasing doses. MG132 concentrations $\leq$2.5 $\mu$M showed no significant differences in viability in LMH cells compared to control cells, and time had no obvious influence at this concentration (Figure 1A); the response to BAY 11-7082 was the same (Figure 1B). LPS at concentrations of 0.1 to 10.0 $\mu$g/mL can't significant inhibited the viability of LMH cells (Figure 1C). In addition, both p65 siRNA

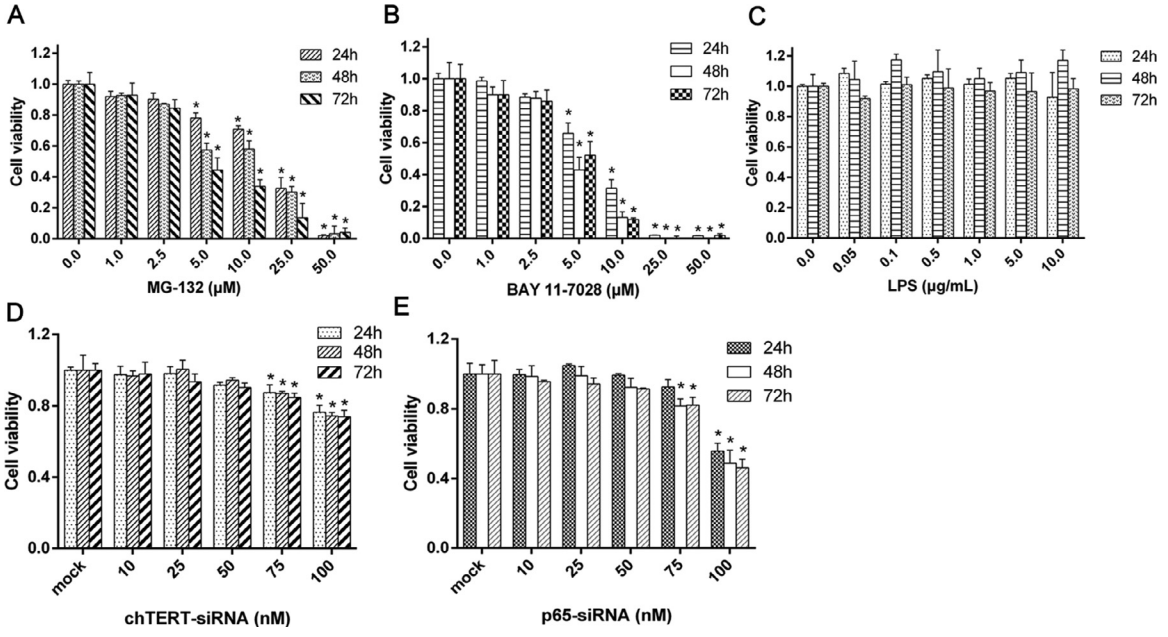


**Figure 1.** Cytotoxic effect of MG132 (A), BAY 11-7082 (B), LPS (C), si-chTERT (D) and si-p65 (E) on LMH cells as determined by MTT assays.

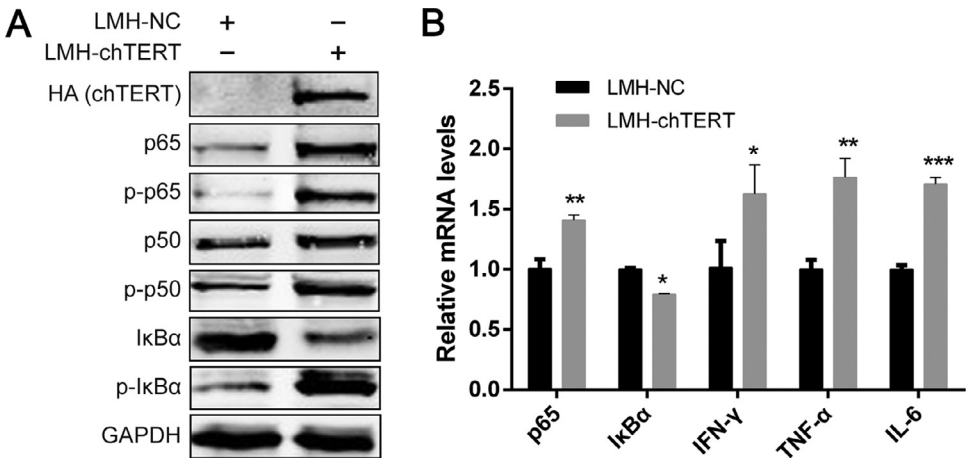


**Figure 2.** chTERT activated the NF-κB signaling pathway. Western blotting and RT-PCR were used to measure the protein and mRNA expression levels, respectively, of chTERT in LMH-chTERT cells and LMH-NC cells.

and chTERT siRNA induced significant cytotoxicity at concentrations ≥75 nM (Figure 1D-E). Therefore, according to the above results and combined with the operation instructions, the optimal concentrations of MG132, BAY 11-7082, LPS and siRNA were 2.5 $\mu$M, 2.5 $\mu$M, 0.1 $\mu$g/mL, and 50 nM, respectively, and follow-up experiments were performed with these concentrations.

## The chTERT Activated the NF-κB Signaling Pathway

The protein and mRNA expression of LMH-chTERT and LMH-NC cells was analyzed by Western blot and RT-PCR, respectively. The results showed that chTERT overexpression could significantly promote the phosphorylation of p65, p50, and IκBα compared with that in the control group and inhibit the IκBα protein and mRNA levels, which suggested that the activity of NF-κB was upregulated (Figure 2). In addition, chTERT also significantly increased the mRNA expression of downstream related inflammatory cytokines, such as INF-γ, TNF-α, and IL-6 ($P < 0.05$). These results indicated that chTERT could further activate the NF-κB signaling pathway and promote the release of inflammatory cytokines.

## The NF-κB Signaling Pathway Regulated the Expression of chTERT and Telomerase Activity

siRNA, activators (LPS), or inhibitors (MG132, BAY 11-7082) were used to interfere with the expression of chTERT or NF-κB in LMH-chTERT cells. The results showed that when the expression of chTERT was inhibited by MG132 or BAY 11-7082, the phosphorylation of p65, p50, and IκBα was inhibited, indicating that the activity of this signaling pathway was significantly reduced ($P < 0.05$) (Figure 3A-B), and the activity of telomerase was also significantly inhibited ($P < 0.05$) (Figure 3C). It was further verified that overexpression of chTERT could upregulate the activity of the NF-κB signaling pathway. Moreover, when the cells were treated with p65 siRNA, not only was the activity of the NF-κB signaling pathway inhibited, but the expression of chTERT was also significantly reduced ($P < 0.05$) (Figure 4A-B). Similarly, the telomerase activity in LMH-chTERT cells was also reduced ($P < 0.05$) (Figure 4C). In contrast, the activity of the NF-κB signaling pathway and the expression of chTERT were both upregulated after LPS treatment ($P < 0.05$) (Figure 3A-B), and telomerase activity was also significantly increased ($P < 0.01$) (Figure 3C). This finding further showed that the β-catenin signaling pathway

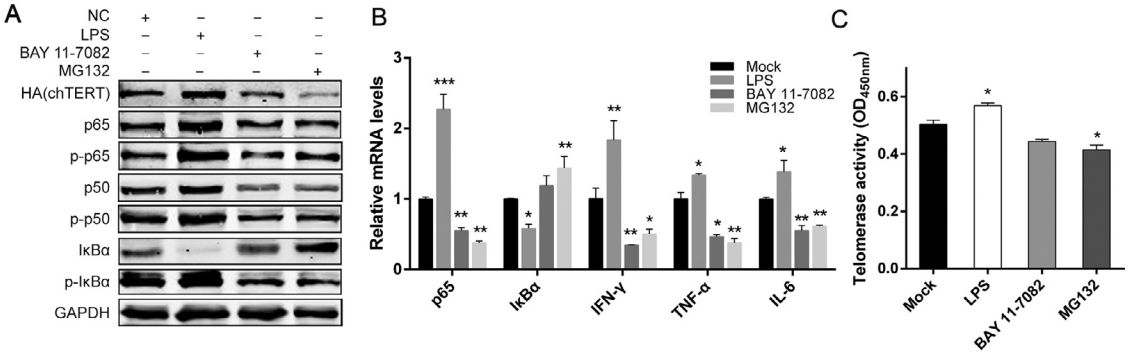


**Figure 3.** The NF-κB signaling pathway could regulate the expression of chTERT. (A) Western blotting and (B) RT-PCR were used to measure changes in chTERT and the NF-κB signaling pathway in LMH-chTERT cells 72 h after treatment with inhibitors (MG132, BAY 11-7082) or activators (LPS). (C) Detection of telomerase activity in LMH-chTERT cells at 72 h after treatment with MG132, BAY 11-7082, and LPS.

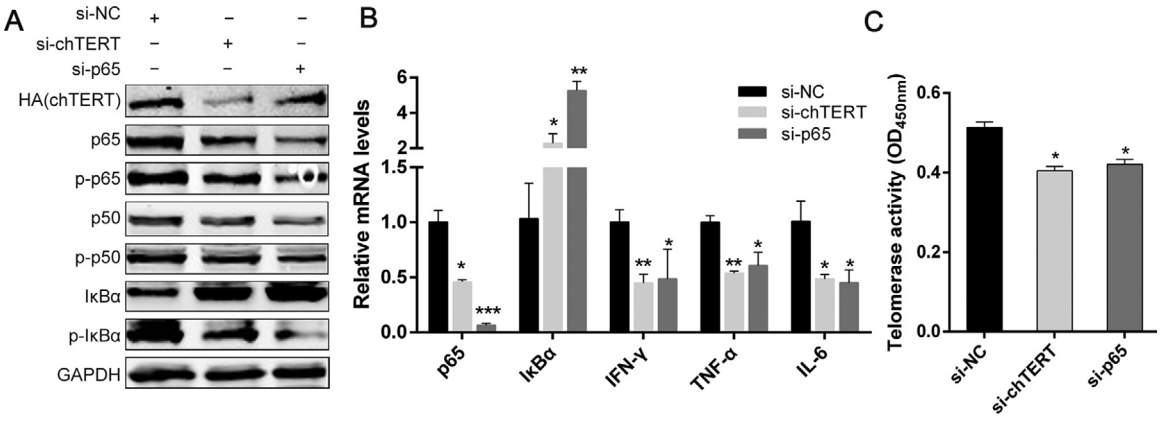


**Figure 4.** The NF-$\kappa$B signaling pathway and chTERT could bidirectionally regulate and promote the expression of each other. (A) Western blotting and (B) RT-PCR were used to measure changes in chTERT and the NF-$\kappa$B signaling pathway 72 h after the siRNA transfection of LMH-chTERT cells. (C) Measurement of telomerase activity in LMH-chTERT cells 72 h after the transfection of si-chTERT or si-p65.

and chTERT gene could bidirectionally regulate and promote expression.

### *The chTERT Mediated LMH Cell Pyroptosis by Regulating the NF-κB Signaling Pathway*

Compared with those in the control group, the transcription levels of Caspase-1, NLRP3, GSDMA, IL-18, and IL-1$\beta$ in LMH-chTERT cells were significantly increased ($P < 0.05$) (Figure 5A), and the protein levels were also significantly increased ($P < 0.05$) except Caspase-11 (Figure 5B), suggesting that chTERT mainly actived the canonical pyroptosis in LMH cells. Significant membrane perforation was observed in LMH-chTERT cells by transmission electron microscopy (Figure 6), suggesting that chTERT could promote LMH cell pyroptosis. To confirm that chTERT could promote pyroptosis and that this effect was achieved by regulating the NF-$\kappa$B signaling pathway, siRNA targeting p65 and chTERT was used to transfect LMH-chTERT cells. The results showed that the mRNA and protein levels of those inflammasomes were significantly downregulated after chTERT or p65 gene knockout ($P < 0.05$) (Figure 7).

## DISSCUSSION

There have been many studies on the hTERT expression regulatory network, and the factors that can bind to its promoter and regulate its expression are very complex. There are binding sites for several transcription factors in the 5' regulatory region of the hTERT gene, including NF-$\kappa$B, Wnt/$\beta$-catenin, TGF-B, Stat, SP1, and c-Myc (Ramlee et al., 2016). As one of the important intracellular pathways, NF-$\kappa$B signaling can regulate the expression of multiple target genes involved in inflammation, the immune response, cell proliferation, differentiation and apoptosis (Yang et al., 2021; Zhou et al., 2021). Several studies have shown that hTERT can promote the nuclear transfer and DNA binding of NF-$\kappa$B p65, and activation of NF-$\kappa$B can also increase the transcription of the hTERT promoter, suggesting that there may be a positive feedback loop between hTERT and NF-$\kappa$B (Chen et al., 2017; Tang et al., 2019). However, this regulatory mechanism has not been confirmed in chickens. It is hypothesized that there is a similar mutual regulatory relationship between chTERT and the NF-$\kappa$B pathway, which needs to be verified. Moreover, there have been few reports on the effects of hTERT or chTERT on pyroptosis.

In the classical NF-$\kappa$B signaling pathway, when cells receive corresponding stimulation, I$\kappa$B dissociates from the p65/p50 dimer and is phosphorylated and degraded under the action of IKK kinase. The nuclear localization signal of the p65/p50 dimer is exposed, which promotes its entry into the nucleus and binding with target genes, inducing the secretion of inflammatory cytokines, including IFN-$\gamma$, TNF$\alpha$, IL-6, and IL-1$\beta$. I$\kappa$B$\alpha$ dissociation is the key to activation of the NF-$\kappa$B pathway (Li and Hu, 2021 ; Sang et al., 2021). In this study, in the chTERT overexpression group, the expression of p65, IFN-$\gamma$, TNF$\alpha$, and IL-6 was significantly increased, I$\kappa$B$\alpha$ was significantly downregulated. Moreover, when the expression of chTERT was inhibited by si-chTERT interference, activation of the NF-$\kappa$B signaling pathway was decreased, which confirmed that chTERT activated the NF-$\kappa$B signaling pathway in LMH cells.

In this study, MG132 and BAY 11-7028 were selected as inhibitors of the NF-$\kappa$B signaling pathway, and LPS was used as an activator to analyze the effect of the NF-$\kappa$B signaling pathway on chTERT. As a commonly used inhibitor of the NF-$\kappa$B pathway, BAY 11-7028 inhibits activation of the NF-$\kappa$B pathway by inhibiting the phosphorylation of I$\kappa$B$\alpha$ and thereby blocking the dissociation of the NF-$\kappa$B dimer and translocation into the nucleus (Liu et al., 2020). MG132 is a proteasome inhibitor that permeates cells and inhibits I$\kappa$B$\alpha$ degradation, thereby inhibiting NF-$\kappa$B activation (Zhao et al., 2020). LPS, a complex of lipids and polysaccharides, mainly acts on Toll-like receptor 4, activates the myeloid differentiation factor pathway to induce the production of inflammatory cytokines, and then activates the NF-$\kappa$B signaling pathway to promote the transcription and expression of inflammatory cytokines (Liu et al., 2021). The expression of chTERT was significantly

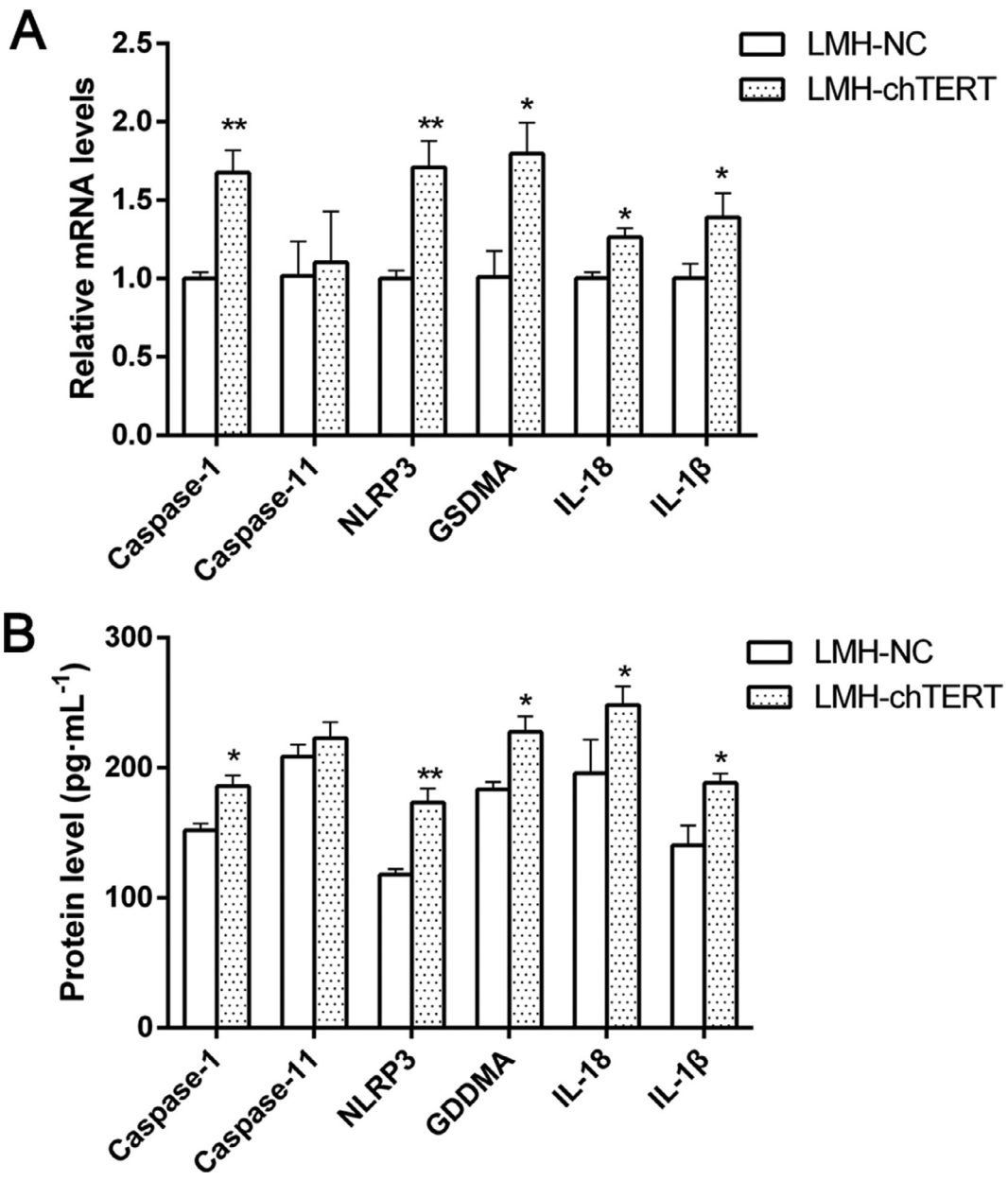


**Figure 5.** chTERT promoted LMH cell pyroptosis. (A) RT-PCR was used to measure the mRNA levels of Caspase-1, Caspase-11, NLRP3, GSDMA, IL-18, and IL-1$\beta$. (B) ELISA was used to measure the protein levels of Caspase-1, Caspase-11, NLRP3, GSDMA, IL-18, and IL-1$\beta$.

downregulated in the inhibitor and p65-siRNA-treated groups but was increased in the LPS-treated group. These results demonstrated that inhibition of the NF-$\kappa$B signaling pathway could reduce the expression of chTERT in LMH cells. In addition, when chTERT expression was inhibited or the activity of the NF-$\kappa$B pathway was inhibited, telomerase activity was reduced, which showed the same pattern of change as that of chTERT expression. Combined with the previous results, these results suggest that the chTERT and the NF-$\kappa$B signaling pathway in LMH cells are mutually regulated and promoted.

Among the functions of hTERT, much attention has been given to its regulation of apoptosis. However, pyroptosis is one of the mechanisms of programmed cell death, and there have been few studies on the regulation of pyroptosis by hTERT or chTERT. The data from the previous part of this study showed that chTERT could regulate the NF-$\kappa$B signaling pathway to promote the expression of inflammatory cytokines. However, pyroptosis is closely associated with the release of proinflammatory factors. Therefore, we hypothesized that chTERT could promote pyroptosis in LMH cells. To verify this hypothesis, RT-PCR and ELISA were used to measure the mRNA and protein levels of cytokines that were closely associated with pyroptosis, respectively. As a result, the expression levels of key factors, such as Caspase-1, the NLRP3 inflammasome, GSDMA and the inflammatory factors IL-18 and IL-1$\beta$, were significantly increased in LMH-chTERT cells.

When pyroptosis occurs, cellular morphology undergoes obvious changes, including cell swelling, plasma membrane rupture, organelle deformation, perforation of the cell membrane (Liu et al., 2017), content efflux, and the formation of a large number of vesicles in pyroptotic cells, which are known as pyroptotic bodies

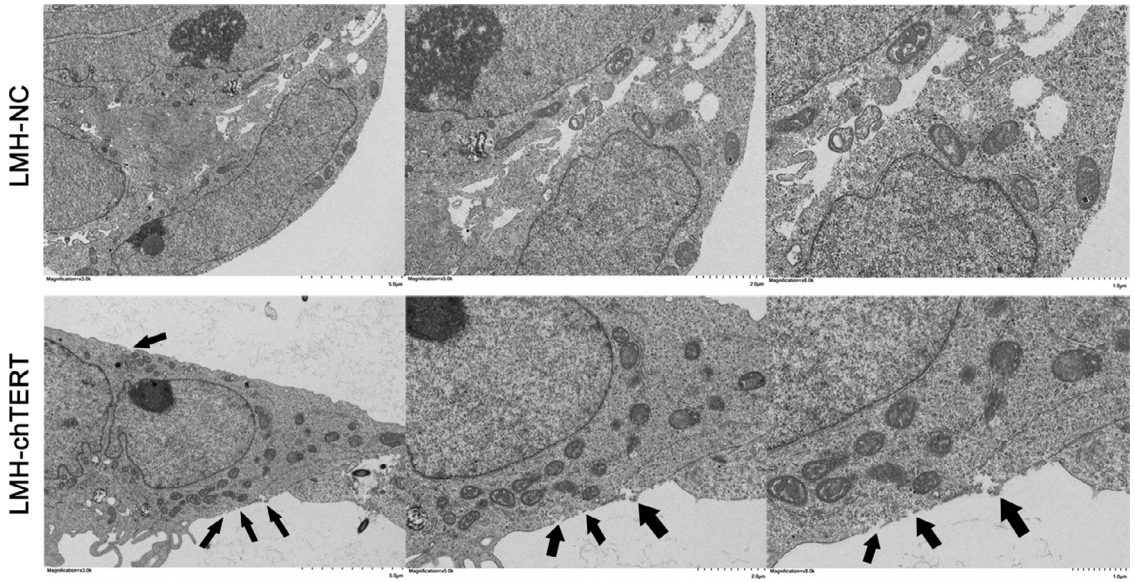


**Figure 6.** Pyroptosis was analyzed by transmission electron microscopy. Membrane perforation (black arrow) was observed in LMH-chTERT cells.

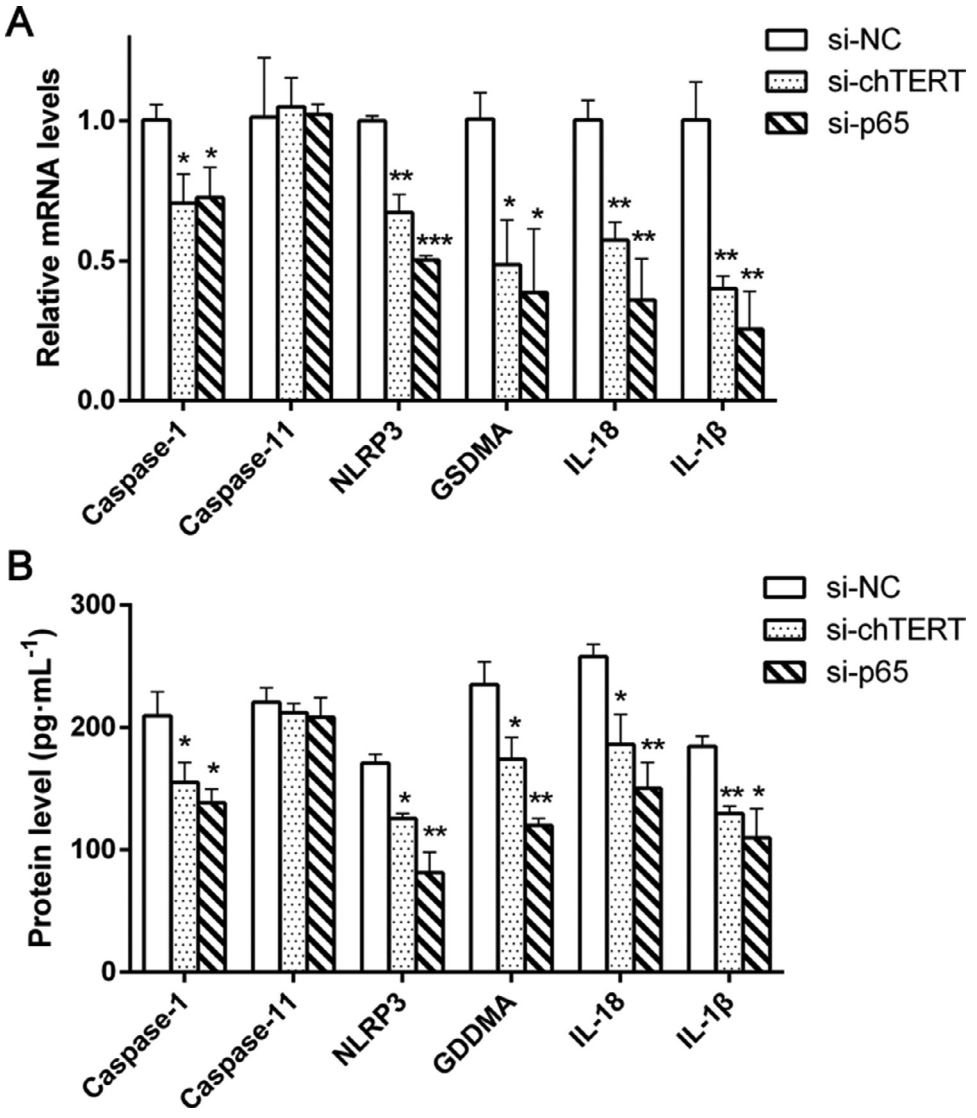


**Figure 7.** chTERT mediated LMH cell pyroptosis by regulating the NF-$\kappa$B signaling pathway. (A) RT-PCR was used to measure the mRNA levels of Caspase-1, Caspase-11, NLRP3, GSDMA, IL-18, and IL-1$\beta$. (B) The protein levels of Caspase-1, Caspase-11, NLRP3, GSDMA, IL-18, and IL-1$\beta$ were measured by ELISA.

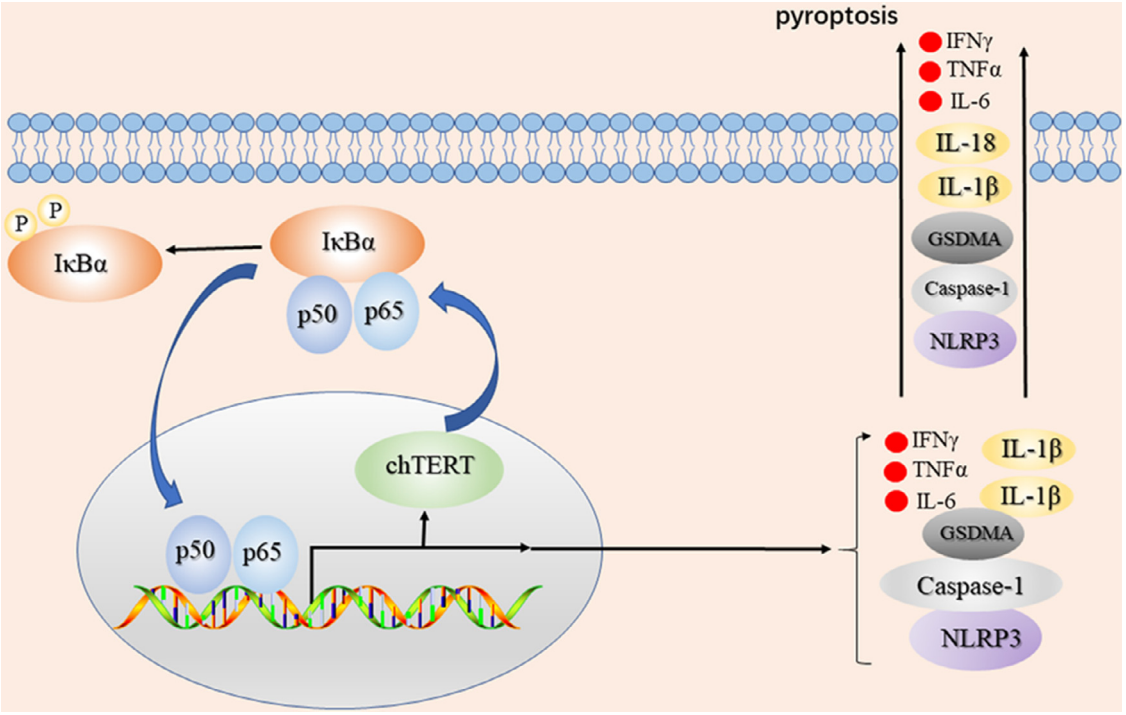


**Figure 8.** The mutual regulation of chicken telomerase reverse transcriptase and NF-κB signaling pathway to mediate pyroptosis in LMH cells.

(Frank and Vince, 2019 ). In this study, we found that chTERT significantly upregulated the expression levels of Caspase-1, NLRP3, GSDMA, IL-18 and IL-1$\beta$, and membrane perforation was observed in LMH-chTERT cells by transmission electron microscopy, which indicated that chTERT overexpression induced pyroptosis. On the other hand, silencing chTERT significantly decreased the expression of these inflammatory factors compared with that in the control group. Moreover, silencing the p65 gene could significantly downregulate the expression level of Caspase-1, NLRP3, GSDMA, IL-18, and IL-1$\beta$, inhibit chTERT-induced pyroptosis, suggesting that NF-κB is a key signaling factor in the regulation of pyroptosis. These results suggested that chTERT mediated the NF-κB signaling pathway to promote the occurrence of pyroptosis in LMH cells.

Activation of the inflammasome is the basis of pyroptosis; in response to external stimuli, NLRP3 can recruit Caspase-1 through adaptor proteins for assembly. Activated Caspase-1 not only mediates the maturation and secretion of inflammatory cytokines (IL-1$\beta$ and IL-18) but also cleaves GSDMD to release the N-terminal domain, thereby inducing pyroptosis (Lei et al., 2018). Caspase-11 drives noncanonical pyroptosis (Xu et al., 2021b) and did not significantly increased in this study. Caspase-1 expression was significantly elevated while caspase-11 did not change significant, suggesting chTERT mainly activation of the canonical pyroptosis pathway. For chickens, GSDMA plays a similar role to GSDMD in humans. And in this study, the expression levels of GSDMA were significantly increased after overexpression of chTERT. These results indicated that chTERT may increase the pore formation in LMH cells via enhancing the activity of gasdermins. The results were consistent with the study that GSDMA can actively promoted the inflammatory response induced by *Salmonella* typhimurium in broilers (Liu et al., 2019).

LMH cells are an avian-derived tumor cell line, which can be used as an in vitro research model for tumor diseases such as avian leukosis and Marek's disease. Our previous research results showed that overexpression of chTERT can up-regulate the Wnt/$\beta$-catenin signaling pathway to shorten the LMH cell cycle, inhibit cell apoptosis, promote cell proliferation and the replication of avian leukosis virus subgroup J (**ALV-J**); conversely, the expression of chTERT can also be upregulated after infected ALV-J (Xiang et al., 2021). This study showed that overexpression of chTERT can promote pyroptosis by up-regulating the NF-κB signaling pathway in LMH cells. The Wnt/$\beta$-catenin and NF-κB signaling pathways are closely related to the occurrence and development of tumors and cell growth and development (Pestana et al., 2017). Therefore, in chickens infected with ALV-J, chTERT may induce the transformation of cell apoptosis to pyroptosis, promote tumor formation and accelerate the deterioration of the disease. Similarly, if chTERT is overexpressed in healthy chickens, it may promote the growth and development of chickens. These speculations are gradually being confirmed in our ongoing in-vivo experiments of chickens.

In summary, chTERT can promote activation of the NF-κB signaling pathway, and conversely, inhibition of the NF-κB signaling pathway can decrease the expression level of chTERT, suggesting that the chTERT and NF-κB signaling pathways are mutually regulated and promoted in LMH cells. In addition, chTERT can

promote the occurrence of pyroptosis by upregulating the NF-$\kappa$B signaling pathway, leading to the release of Caspase-1, NLRP3, GSDMA, IL-18, and IL-1$\beta$ (Figure 8).

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## DISCLOSURES

The authors declare no conflicts of interest.

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