ISSN 2706-6819 Vol.7, Issue 4: 50-58, DOI: 10.25236/IJFM.2025.070408

Regulation of Autophagy in Mouse Embryonic Palatal Mesenchymal Cells by Dexamethasone through the PI3K/AKT Pathway

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Abstract: *This study aims to investigate the mechanism by which dexamethasone regulates autophagy in mouse embryonic palatal mesenchymal cells, thereby providing a theoretical basis for the prevention of cleft palate. In vitro, the 14.5-day palatine mesenchymal cells of C57BL/6J mouse embryos were extracted and cultured in vitro and the 14.5-day palatine mesenchymal cells were randomly divided into control (CON) group and dexamethasone (DEX) groups according to the intervention conditions. The number of autophagosomes or autophagolysosomes in each group was observed by transmission electron microscopy. Western blot was used to detect the protein expression changes of PI3K, p-PI3K, AKT, p-AKT, mTOR, LKB1, p-LKB1, p38MAPK, p-p38MAPK and autophagy markers LC3II/I, P62 and Beclin 1. Transmission electron microscopy results showed that the number of autophagosomes/autophagolysosomes was significantly reduced in the DEX group compared with the CON group (P<0.05). Western blot results showed that compared with the CON group, Beclin 1 was reduced in DEX group, p62 was increased (P<0.05), and there was no significant change in LC3II/I expression (P>0.05); PI3K/AKT/mTOR signaling pathway was significantly inhibited in DEX group (PI3K, p-PI3K, AKT, p-AKT, mTOR expression was decreased, P<0.05), and LKB1 signaling pathway and p38MAPK signaling pathway were significantly activated ((LKB1, p-LKB1, p38MAPK, p-p38MAPK, p-p38MAPK, P<0.05). DEX may be activated by inhibiting the PI3K/AKT/mTOR pathway, and p38MAPK pathway inhibits autophagy of mouse embryonic palatal mesenchymal cells.*

Keywords: *Dexamethasone; Cleft Palate; PI3K/AKT/Mtor/LKB1/P38mapk Signaling Pathway; Cell Autophagy*

1. Introduction

Non-syndromic orofacial clefts (OFCs) are congenital malformations that affect approximately 1.7 per 1000 newborns globally[1]. The most common forms of OFCs can be classified as cleft lip only (CL), cleft lip with cleft palate (CLP) or cleft palate only (CP)[2]. The incidence rate of non-syndromic cleft lip and palate in China is about 1.38‰~1.67‰[3]. This disease has had a profound impact on patients' eating, sucking, pronunciation, listening function, teeth arrangement and mental health, so it requires a comprehensive therapeutic intervention of multidisciplinary combination. The causes of CLP are numerous, including genetic and environmental factors[4].

Dexamethasone (DEX) is a common glucocorticoid. In clinical practice, excessive use of pregnant women may induce fetal cleft palate, and the dose and potential risks need to be strictly evaluated[5]. Therefore, it is of great clinical significance to in-depth study of the mechanism of DEX-induced cleft palate. Previous studies of this research group found that activating cell autophagy can significantly reduce the occurrence of cleft palae in mouse embryos, but the specific mechanism is still unclear. Therefore, this study focuses on the changes of several signaling pathways (PI3K/AKT/mTOR/ LKB1/p38MAPK) that mainly regulates cell autophagy under the influence of DEX, and conducts research on the molecular mechanism of DEX affecting mouse embryonic palatal mesenchymal (MEPM) cells, further providing prospective theoretical basis for the feasibility of reversing DEX

ISSN 2706-6819 Vol.7, Issue 4: 50-58, DOI: 10.25236/IJFM.2025.070408

inducing cleft palae and providing useful clues for the prevention and treatment of cleft palae.

2. Materials and methods

2.1 Experimental Animals

8-10-week-old SPF-grade C57BL/6J mice were selected and raised in the SPF-grade animal laboratory at the Laboratory of the Laboratory Animal Center of ZunYi Medical University. License number: SYXK (Guizhou) 2021-0004. Laboratory ambient temperature (25±2)℃, relative humidity 20%-30%, and conventional feeding under manual regulation for 12 hours of day and night cycle conditions. Male and male mice (body mass: females above 18 g, males above 20 g) were combined at night. In the morning of the next day, the female mice had vaginal ducts and were predicted to have conception and marked as mouse embryos for 0.5 days (Embryonic Day 0.5, ED0.5). Those who gained≥2g at ED10.5 were determined to be pregnant mice.

All experimental animal operations in this study were reviewed and approved by the Animal Welfare Ethics Committee of ZunYi Medical University (No: Lunshen [2021] No.2-504). The experimental process is subject to the International Association of Veterinary Editors' Consensus on Author Guidelines on Animal Ethics and Welfare and local and national regulations. The experimental animals were given anesthesia under inhalation of carbon dioxide and sacrificed with neck, in order to minimize their pain and death.

2.2 Drugs and reagents

Trypsin, DMEM medium, 10% fetal bovine serum, penicillin-streptomycin dual anti-anti-solution and ECL luminescent chromogenic solution were purchased from(Thermo Fisher, USA); DEX, neutral protease, BCA protein assay kits and DAB were purchased from (Solarbio, China); RIPA lysate was purchased from (beyotime, China); PBS, TBST, PVDF membranes, P62, Beclin1, LC3 and Actin monoclonal antibodies were purchased from (Servicebio, China); mTOR monoclonal antibodies were purchased from (Abcom, USA); SABC immunohistochemistry kits were purchased from (Boster Bio, USA); GADPH, PI3K, p-PI3K, AKT, p-AKT, LKB1, p-LKB1, p38MAPK monoclonal antibodies, horseradish peroxidase-labeled sheep anti-rabbit secondary antibody and horseradish peroxidase-labeled sheep anti-mouse secondary antibody were purchased from (Proteintech, USA).

2.3 Cell culture and grouping

At ED14.5, pregnant mice were euthanized. Under sterile conditions, the palatine process was dissected and separated from the oral cavity of the fetal rat under aseptic microscope, and digested overnight in neutral protease solution at 4 °C (or the digestion time was greater than 12hours). The next day, centrifuged to separate epithelial and mesenchymal tissues and digested with 0.25% trypsin at 37 °C for 5 minutes to obtain primary cells. The cells were cultured in DMEM medium, which contained 10% fetal bovine serum and penicillin-streptomycin biantibacterium solution. Thereafter, it will be passed down every 2-3 days. MEPM cells cultured to the third generation were randomly divided into CON group and DEX group (1 μmol/LDEX intervention).

2.4 Immunohistochemistry experiments

This experiment used anti-Vimentin antibodies and anti-PCK antibodies to identify primary MEPM cells. After identification as mesenchymal cells, it was used in the following experiments.

2.5 Transmission electron microscopy

When MEPM cells were cultured to the third generation, they were randomly divided into control group and DEX group according to the intervention method. Tryptic enzyme digestion, low temperature centrifugation, discarded supernatant, fixed by electron microscopic fixation, rinsing by PBS, and fixed by 1% osmium acid; gradient dehydration, embedding, ultra-thin sections, double staining of uranium lead, observation and photography under transmission electron microscopy, and the number of autophagosomes/autophagolysosomes in each group was recorded.

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2.6 Western Blot analysis

The third generation of MEPM cells were extracted after regular culture and DEX intervention. The concentration of each histone was measured by BCA method. Then adjust the concentration of each histone to 1 g/L. After adding an appropriate amount of 5×SDS loading buffer, bathe at 100℃for 10 min to denature the protein, aliquot, label, and store in a -20℃refrigerator. The electrophoresis gel was configured, and each group of samples was added and the Marker was electrophoresis and transferred to the membrane. The primary antibody at an appropriate concentration was added. After incubating overnight at 4℃ shaker, the primary antibody was washed three times with TBST, and the horseradish peroxidase-labeled sheep anti-rabbit secondary antibody (1:5 000) was incubated for 1 h at room temperature. After washing TBST, the expression of target proteins in each group was detected by the ECL chromogenic development system.

2.7 Statistical Methods

All statistical analyses in this study were performed using GraphPad Prism 9 software. All values are expressed as $\overline{X}\pm s$. The data of the normal distribution were compared with the differences between the two groups using independent sample t test. The difference was considered statistically significant at $P < 0.05$.

3. Results

3.1 DEX inhibits autophagy in MEPE cells

There are a certain number of vacuole-like structures in the CON group, including autophagosomes formed by cytoplasmic components, with bilayer or multilayer membrane structures, or autophagolysosomes containing degraded cytoplasmic components and having a monolayer membrane structure[6](see Figures 1A and A'); compared with the CON group, the number of autophagosomes/ autophagosomes in the DEX group was significantly reduced (see Figures 1B and B'), and the difference was statistically significant ($P<0.05$) (see Figure 1C). To further verify that DEX can inhibit autophagy in MEPE cells, Western blot detected the expression of autophagy-related proteins. Compared with the CON group, there was no significant difference between the expression of LC3II/I in the DEX group and the normal group ($P>0.05$), while Beclin1 was significantly reduced, and the difference between the expression of p62 was significantly upregulated ($P<0.05$). Our results show that DEX inhibits autophagy in MEPE cells (see Figure 2).

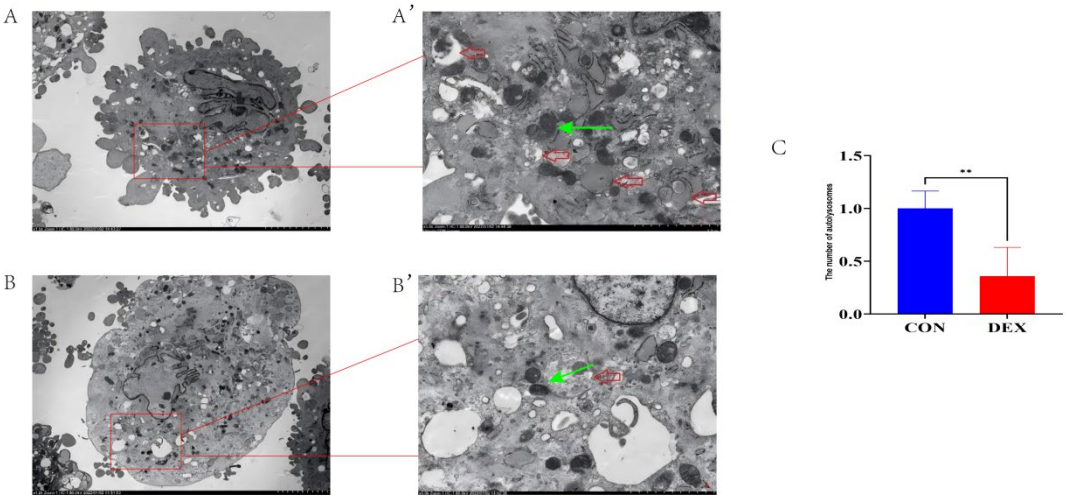

*Figure 1 DEX inhibits autophagosome/autophagolysosome expression in MEPE cells. Panels A and A' are the CON groups, Panels B and B' are the DEX groups, and Panels C are the statistical results of autophagosomes/autophagolysosomes in the CON and DEX groups (compared with the control group, the expression of autophagosomes/autophagolysosomes in the DEX group was significantly reduced, **indicates P<0.01); (The red arrow indicates autophagosomes or autophagolysosomes, and the green arrow indicates mitochondria; Panels A and B scale bar = 2 microns; Panels A' and B' scale bar = 500 nanometers).*

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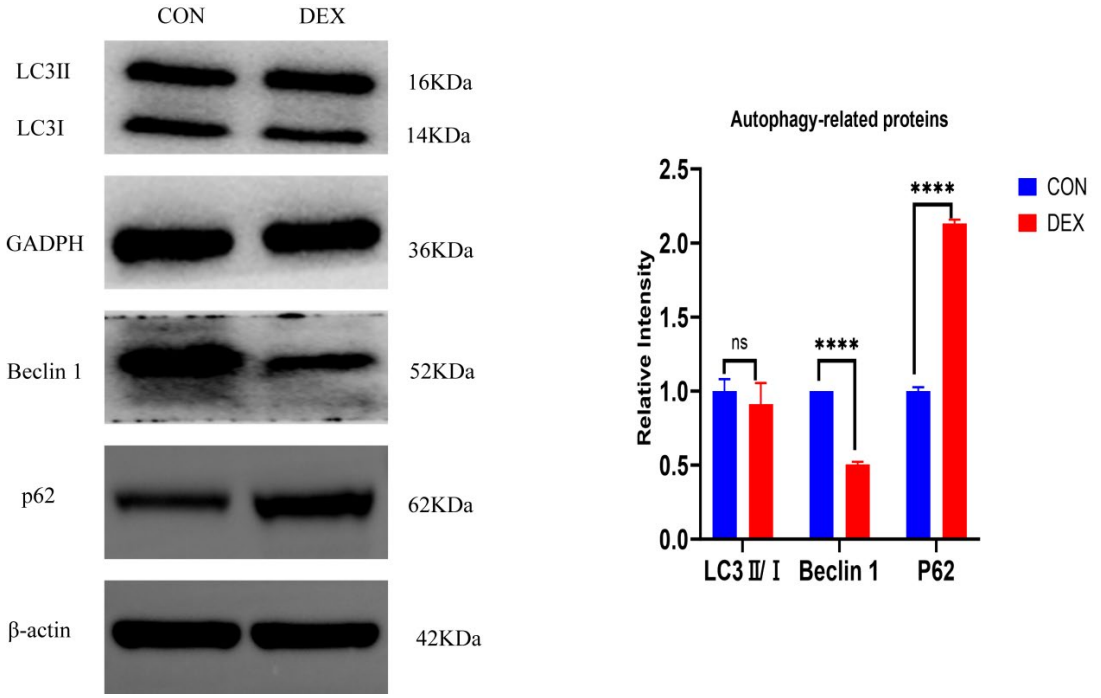

*Figure 2 DEX inhibits the expression of autophagy-related proteins in MEPE cells. Compared with the CON group, the expression of Beclin 1 proteins was significantly reduced in the DEX group, P62 proteins was significantly increased, and LC3 proteins was not different ($\bar{X}$±s,n=3) (ns indicated no significant difference, ****P<0.0001).*

3.2 DEX inhibition of PI3K/AKT/mTOR pathway

In order to study the molecular mechanism by which DEX inhibits autophagy in MEPE cells, we focused on the PI3K-AKT-mTOR pathway because it plays a key role in autophagy regulation[7]. Therefore, we examined the protein expression levels of PI3K, p-PI3K, AKT, p-AKT, mTOR after DEX-treated cells. Compared with the CON group, the levels of PI3K, p-PI3K, AKT, p-AKT, and mTOR proteins in the DEX group were significantly reduced, and the differences were statistically significant (P<0.05). These findings suggest that the PI3K-AKT-mTOR pathway is involved in DEX inhibition of autophagy in MEPE cells (see Figure 3).

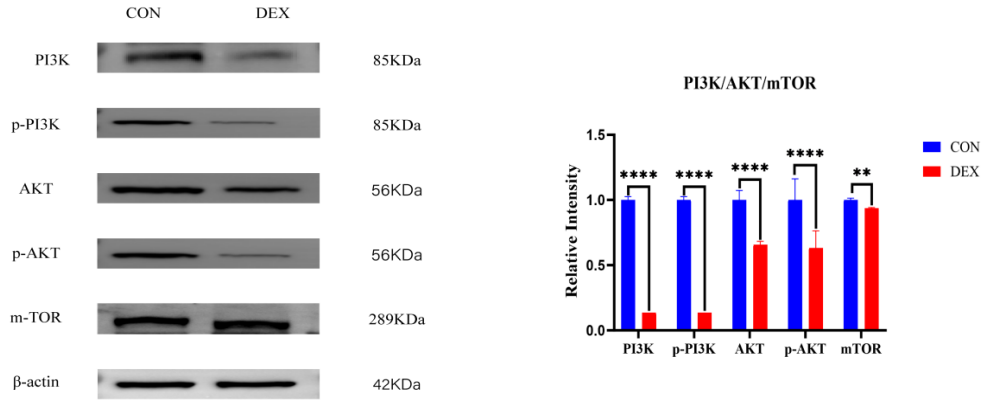

*Figure 3 DEX inhibits the expression of proteins in the PI3K/AKT/mTOR pathway in MEPE cells. Compared with the CON group, the expression of PI3K,p-PI3K,AKT,p-AKT,mTOR proteins in the DEX group was significantly reduced ($\bar{X}$±s, n=3).(**P < 0.01, ****P < 0.0001).*

ISSN 2706-6819 Vol.7, Issue 4: 50-58, DOI: 10.25236/IJFM.2025.070408

3.3 DEX activates LKB1 path

In addition, after reviewing the literature, LKB1 is also involved in the autophagy process of cells, so we tested changes in protein expression of the LKB1 pathway after DEX treatment. We examined the protein expression levels of LKB1 and p-LKB1 after DEX-treated cells. We observed that compared with the CON group, the levels of LKB1 and p-LKB1 proteins in the DEX group increased significantly, and the differences were statistically significant (P<0.01). The above research results show that DEX can activate the LKB1 pathway involved in inhibiting autophagy in MEPE cells (see Figure 4).

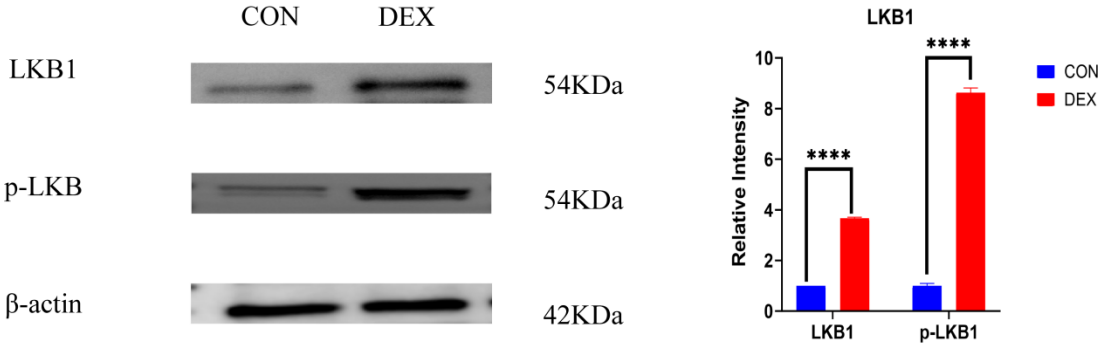

*Figure 4 DEX activates the expression of LKB1 pathway protein in MEPE cells. Compared with the CON group, the expression of LKB1 and p-LKB1 protein in the DEX group was significantly increased ($\bar{X} \pm s$,n= 3). (****P< 0.0001).*

3.4 DEX activates p38MAPK pathway

In the same way, in order to study the mechanism of autophagy in DEX-acting MEPE cells, we also examined the changes in the p38MAPK pathway after DEX treatment, and found that activation of p38MAPK-related pathways can inhibit autophagy activity. We examined the protein expression levels of p38MAPK and p-p38MAPK after DEX-treated cells. We observed that compared with the CON group, the levels of p38MAPK and p-p38MAPK proteins in the DEX group increased significantly, and the difference was statistically significant (P<0.01). The above research results show that DEX can activate the p38MAPK pathway involved in inhibiting autophagy in MEPE cells (see Figure 5).

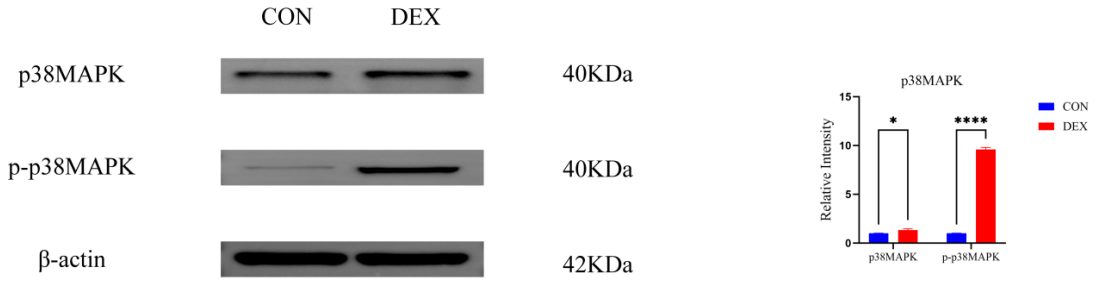

*Figure 5 DEX activates the expression of p38MAPK pathway protein in MEPE cells. Compared with the CON group, the expression of p38MAPK and p-p38MAPK protein in the DEX group was significantly increased ($\bar{X} \pm s$,n= 3). (*P<0.05, ****P < 0.0001).*

4. Discussions

Cleft lip and palate are the result of a complex gene-environmental interaction, but insufficient understanding of environmental risk factors hinders the development of prevention strategies[8]. At present, animal models have shown that DEX can induce cleft palate in animal embryos , and it was found on the cell level that DEX can promote the apoptosis level of MEPE cells, resulting in failure of bilateral palatine fusion[9]. In previous studies, this research team found that DEX can inhibit the proliferation of MEPE cells by affecting Shh signaling in MEPE cells [10], and found that Shh signaling in MEPE cells is dependent on primary cilia [11], and the addition of Shh signaling pathway activator can induce autophagy in MEPE cells [12]. Therefore, we want to explore the changes in the autophagy

ISSN 2706-6819 Vol.7, Issue 4: 50-58, DOI: 10.25236/IJFM.2025.070408

mechanism of MEPE cells under the action of dexamethasone.

Autophagy is a key catabolism pathway in response to metabolism and energy stress in eukaryotes [13]. Autophagy can be divided into three different forms according to the different cellular functions and morphology of cytoplasmic substances, namely macroautophagy, microautophagy and chaperone-mediated autophagy. Among them, macroautophagy (hereinafter referred to as autophagy) is the most widely studied form of autophagy[14]. Various human diseases, such as cancer, metabolic diseases, immune disorders and neurodegenerative diseases, are closely related to autophagy disorders [15]. Shu et al. studied in all-trans retinoic acid-induced cleft palate in mice and found that autophagy/beclin 1 regulator 1 (AMBRA 1)-mediated autophagy and apoptosis associated with epithelial-mesenchymal transformation. NONMMUT034790.2-LEF1-AMBRA1trans-regulatory network may be an important mechanism of action for dysfunctional palate fusion [16]. Chen et al. found that chloroquine can regulate the proliferation and apoptosis of mouse embryonic palatine cells by activating P53. At the same time, autophagy triggered by ROS/ERK signaling protects nicotine-induced apoptosis of mouse embryonic palatine cells, thus affecting the biological characteristics of mouse embryonic palatine development [17, 18]. Luo et al. found that Lhx6 maintains mitochondrial homeostasis by regulating PINK1/Parkin-mediated mitochondrial autophagy and MAPK signaling pathway. Retinoic acid destroys mitochondrial homeostasis through transcriptional inhibition of Lhx6, which in turn leads to disorders in the proliferation and migration of human embryonic palatine mesenchymal cells, and ultimately triggers cleft palate [19]. However, research results show that MEPM cells can maintain their stem cell properties through PTEN/AKT/mTOR autophagy signaling to prevent the occurrence of cleft palate [20].

In this study, the autophagic vesicle structure that can be observed under transmission electron microscopy is consistent with the results observed by other scholars using transmission electron microscopy[21]. This shows that under normal physiological conditions, MEPE cells involved in normal palatal metabolism have an autophagy pathway. However, the number of autophagosomes/ autophagolysosomes in MEPE cells was significantly reduced at the current concentration of DEX and time of action. At the same time, Western Blot results showed that compared with the CON group, DEX-treated MEPE cells significantly reduced the expression of the autophagy marker Beclin1 protein at 24h, while the expression of p62 protein was significantly higher than that of the control group, indicating that DEX-treated 24h can inhibit the autophagy activity of MEPE cells. At the same time, studies have found that after DEX treatment of MC3T3-E1 cells, the LC 3-II/LC 3-I ratio and Beclin 1 protein content were significantly reduced, and the autophagy activity was inhibited. However, when Li et al. explored the mechanisms of DEX inducing osteoblast damage, they found that after 24 hours of treatment with 1 μmol/L and 10 μmol/L dexamethasone, the expression of Beclin-1 and LC3 was significantly upregulated (P<0.05), and the results showed that DEX significantly promoted autophagy[22]. Studies have also found that resveratrol can enhance the expression of SIRT1 in osteoblasts treated with DEX (1μmol/L DEX) and protect osteoblasts by regulating the PI3K/AKT/mTOR signaling pathway to upregulate mitochondrial autophagy levels [23]. The inconsistency of the above study results may be related to different DEX doses and their effects on different cell types. Therefore, we speculate that DEX regulates autophagy in MEPE cells.

As a complex catabolism process, autophagy is regulated by a variety of signaling pathways, such as PI3K/AKT/mTOR, LKB1, and p38MAPK. The PI3K/AKT/mTOR signaling pathway consists of phosphatidylinositol-3-kinase (PI3K), protein serine/threonine kinase AKT (also known as protein kinase B), and mTOR. This signaling pathway exists in almost all cell types and plays a crucial role in the cell. It is involved in regulating a variety of life activities, including cell metabolism, proliferation, survival, growth and blood vessel formation [24]. For the PI3K signaling pathway, the most critical gene is PI3K [25], and activation of mTOR-related pathways can promote cell autophagy [26, 27]. Through the study of stem cell characteristics of MEPE cells, Shi et al. found that MEPE cells can maintain their stem cell state through the PI3K/AKT/mTOR autophagy signaling pathway and prevent the occurrence of cleft palate[20] . Studies have found that genipinol activates autophagy through the GLP-1 R/PI3K/AKT/mTOR pathway, thereby improving DEX-induced apoptosis of osteoblasts [28]. Lycopene inhibits the AKT signaling pathway, activates mitochondrial autophagy, and alleviates renal fibrosis [29]. GN inhibits autophagy mild cerebral ischemia-reperfusion injury through activation of the PI3 K/AKT/mTOR signaling pathway[30]. In addition, amylin amethrin reduces osteoarthritis by reducing TNFSF 11 expression in cartilage and inhibiting PI3K/AKT/mTOR signaling activates mitochondrial autophagy [31].This study found that in the PI3K/AKT/mTOR signaling pathway, after DEX administration, the protein expression levels of PI3K, p-PI3K, AKT, p-AKT, and mTOR were significantly decreased in the DEX group compared with the CON group. This demonstrates that DEX intervention inhibits the PI3K/AKT/mTOR signaling pathway and activates cellular autophagy.

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Liver kinase B1 (LKB1) is an important serine/threonine kinase and is a tumor suppressor gene found in patients with multiple digestive tract mellitus syndrome [32]. It regulates glucose and lipid metabolism, as well as other cellular functions such as cell autophagy and cell polarity[33]. After activation of LKB1, AMPK and p53 signaling pathways can be activated by homeostasis, and the mTOR pathways negatively regulate the mTOR pathway, which can promote autophagy[34]. It has been found that Metrnl improves diabetic cardiomyopathy through inactivation of cGAS/STING signaling dependent on LKB 1/AMPK/ULK 1-mediated autophagy [35]. LKB1 activators can prevent renal fibrosis through LKB 1/AMPK-mediated autophagy and mitochondrial homeostasis pathways [36]. In this study, it was found that compared with the CON group, the protein expression of LKB1 and p-LKB1 in the DEX group increased, suggesting that the LKB1 signaling pathway is involved in DEX-induced autophagy of MEPE cells. Although the interaction relationship between LKB1 and mTOR in MEPE cells was not discussed, the expression of mTOR showed a downward trend after DEX induction, while LKB1 was in an upward trend. It can be seen that the expression of LKB1 and mTOR was opposite in the process of autophagy in MEPE cells. Activation of the LKB1 pathway will homeopathically activate the AMPK pathway resulting in inhibition of mTOR activity, thereby activating cell autophagy [34].

p38MAPK signaling factor is a class of serine/threonine protein kinases present in intracellular mitogen-Activated Protein Kinases (MAPKs) [37]. It is worth noting that glucocorticoids have a complex role in the p38 signaling pathway. DEX can not only affect the expression levels of β-globin and cyclooxygenase-2 (COX-2) by inhibiting p38 activity, but also inhibit cell autophagy; glucocorticoids can also directly activate the p38 pathway, causing the gene expression of h-Sgk to inhibit autophagy [38, 39]. For example, in microglia, lipopolysaccharides cause phosphorylation of p38 by activating p38, thereby inhibiting autophagy of microglia [40]. Studies have shown that PIKfyve and p38 MAPK cooperate to regulate lysosomal homeostasis, and their combination inhibits synergistically blocking autophagy to reduce cancer cell viability in vitro and in vivo [41]. In addition, studies have found that berberine enhances autophagy and inhibits iron apoptosis by inhibiting the JNK-p38 MAPK pathway, further reduces β-amyloid plaque deposition, inhibits inflammatory response, and improves neuronal damage, thereby alleviating Alzheimer's disease [42]. In this study, it was found that after DEX treatment, compared with the CON group, the protein expression of p38MAPK and p-p38MAPK in the DEX group showed an upward trend. After activation of the p38MAPK signaling pathway, downstream mTOR can be activated, but mTOR is mainly regulated by PI3K/AKT and other pathways. This pathway inhibits mTOR, so the autophagy state of glucocorticoids did not change after activation of p38MAPK. In mammals, the ULK/Atg 1 family consists of two main members ULK1 and ULK2[43]. ULK1 and ULK2 are also a class of silk/threonine kinases, known for their core role in evolutionary conservatism in the autophagy pathway, and can serve as biological varistors to precisely regulate the cell's response to internal and external environmental signals [44]. Studies have found that AMPK and mTOR can regulate autophagy by direct phosphorylation of ULK1 [45]. Therefore, we guess that DEX also had an impact on the expression of ULK1 in this experiment, which caused the autophagy of MEPE cells to be inhibited.

In summary, after DEX treatment, the PI3K/AKT/mTOR signaling pathway in MEPE cells was significantly inhibited. Although autophagy was partially activated, activation of LKB1 and p38MAPK pathways resulted in an inhibitory state of overall autophagy levels in MEPE cells. This effect may be related to complex regulation between multiple signal paths. DEX directly interferes with or negatively regulates the MAPK pathway through binding to glucocorticoid receptors and leads to downregulation of Beclin1 expression, thereby reducing autophagy activity. However, this study has only been initially verified in in vitro cell models and has not fully disclosed the interaction mechanism between the three signaling pathways of PI3K/AKT/mTOR/LKB1/p38MAPK. Future research needs to further explore the network regulation relationship of these signaling pathways and their coordinated regulatory mechanisms on cell autophagy, and verify relevant findings in in vivo models. In addition, it is necessary to deeply analyze the specific molecular mechanisms of DEX regulating the association between autophagy and apoptosis in MEPE cells, and provide a theoretical basis for the prevention and treatment of cleft palate.

5. Conclusion

Dexamethasone may significantly inhibit autophagy of mouse embryonic palatine mesenchymal cells by inhibiting the PI3K-AKT-mTOR pathway and activating the LKB1 and p38MAPK pathways.

ISSN 2706-6819 Vol.7, Issue 4: 50-58, DOI: 10.25236/IJFM.2025.070408

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