The YAP/HIF-1 α /miR-182/EGR2 Axis is Implicated in Asthma Severity by Control of Th17 Cell Differentiation

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Research

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Abstract

Background: Asthma is a heterogeneous chronic inflammatory disease of the airways, with reversible airflow limitations and airway remodeling. T helper 17 (Th17) cells play an important role in the pathogenesis of allergic asthma. However, there is hitherto little data about signaling pathways controlling Th17 cell differentiation in asthma. The aim of this study was to ascertain whether the YAP/HIF-1 α /miR-182/EGR2 axis underpins Th17 cell differentiation and asthma severity.

Methods: The study included 29 pediatric patients with asthma, 22 healthy volunteers, ovalbumin (OVA)-induced murine asthma models, and mouse naive CD4⁺ T. The subpopulation of Th17 cells was examined by flow cytometry. The level of IL-17A was determined by ELISA method. ChIP-qPCR assay and dual-luciferase reporter gene assay were performed to examine interaction between HIF-1 α and miR-182, miR-182 and EGR2.

Results: YAP, HIF-1 α , and miR-182 were found to be up-regulated but EGR2 was down-regulated in human and mouse peripheral blood mononuclear cells (PBMCs) in the context of asthma. Abundant expression of YAP and HIF-1 α promoted miR-182 expression and then inhibited EGR2, a target of miR-182, thus enhancing Th17 differentiation and deteriorating asthma and lipid metabolism dysfunction. In addition, *in vivo* findings revealed that over-expression of EGR2 undermined the promoting effect of the YAP/HIF-1 α /miR-182 axis on asthma and lipid metabolism dysfunction.

Conclusion: These results shed light on that the activation of the YAP/HIF-1 α /miR-182/EGR2 axis may promote Th17 cell differentiation, exacerbate asthma development, and aggravate lipid metabolism dysfunction, providing a potential therapeutic target in asthma.

Background

Asthma is a frequently occurring disease of the airways, affecting about 300 million people worldwide [1]. Asthma incidence is higher in high-income countries, while asthma-related mortality is predictably higher in lower-income countries [2]. Moreover, although the global prevalence of asthma is about 4–5%, this figure can vary as much as 21-fold in different countries [2]. The vast majority of patients with asthma present with concomitant rhinitis, which can amplify the risk for developing asthma, and exhibit heightened bronchial hyperresponsiveness and heightened reactivity to a variety of stimuli [3]. Current day management of asthma primarily focuses on alleviating disease severity and choosing the appropriate medical therapy to control symptoms and reduce the risk of exacerbations; however, some patients still experience acute exacerbations of symptoms and a loss of disease control [4, 5], highlighting the urgent need to fully elucidate the underlying pathogenesis and develop more efficacious treatment regimens to better tackle asthma [6].

Recent data found that Yes-associated protein (YAP), a downstream target of the Hippo pathway, is implicated in the generation and maintenance of cancer-associated fibroblasts and vascular smooth muscle cell differentiation [7]. YAP holds significant potential as an anticancer immunotherapeutic target owing to the fact that YAP deficiency induces dysfunctional Tregs unable to suppress anti-tumor immunity or promote tumor growth [8]. Silencing YAP has also been shown to facilitate airway smooth muscle cells proliferation, migration and contraction induced by sphingosine-1-phosphate in asthma [9]. In addition, YAP can bind to

hypoxia-inducible factor 1 α (HIF-1 α) and maintain its protein stability, thus promoting hepatocellular carcinoma cell glycolysis under hypoxic stress [10]. During the induction of asthma, treatment with HIF-1 α inhibitor decreases eosinophilia in bronchoalveolar lavage, lung parenchyma and total lung inflammation [11]. Meanwhile, HIF-1 α has also been shown to promote the expression of microRNA-182 (miR-182) [12]. This is notable as miR-182 is also up-regulated during T-helper 17 (Th17) cell differentiation [13]. Accumulating studies have revealed the correlation between activated Th17 cells and the progression of asthma [14–16]. Moreover, early growth response 2 (EGR2), known as a transcription factor negatively regulating T-cell activation [17], has been reported to reduce Th17 cell differentiation [18]. In addition, reduction of high-density lipoprotein cholesterol (HDL-C) has been linked to increased Th17 cells [19]. More importantly, decreased HDL-C levels have been found in children with asthma [20]. Therefore, we sought to determine if YAP1 and HIF-1 α function through a pathway that involves low HDL-C-induced increased Th17 cells in the pathogenesis of pediatric asthma. These results could potentially imply a novel link between HIF-1 α , miR-182, EGR2, and Th17 cells. Therefore, we investigated if miR-182 and EGR2 were involved in the asthma pathway.

Results

YAP, HIF-1 α and miR-182 Are up-regulated and HDL-C levels are decreased in Asthma

Aiming to screen the possible genes implicated in the development of asthma, a differential gene expression analysis was performed on the asthma-related GSE27876 dataset retrieved from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/gds>) with the help of the R package Limma (<http://www.bioconductor.org/packages/release/bioc/html/limma.html>). The GSE27876 dataset (comparing peripheral blood cells from mild and severe asthma that were selected from patients classified into the asthma treatment step 4, in accordance with the criteria described in the Global Initiative for Asthma) comprised of 5 normal samples and 10 asthma samples, and employed peripheral blood as the sample type. With the cutoff criteria ($|\log FC| > 1.5$, and $p < 0.05$), a total of 331 differentially expressed genes (DEGs) were screened (Fig. 1A, Supplementary Table 1). In addition, 318 human transcription factors were identified from the Cistrome database (<http://cistrome.org/>). Following Venn diagram analysis of the 331 DEGs and 318 human transcription factors, 5 transcription factors were found at the intersection, namely H2AFX, NOTCH1, RUNX1T1, USF1, and YAP1 (Fig. 1B, Supplementary Table 2). Subsequently, the protein-protein interaction (PPI) among the aforementioned 5 transcription factors and their related genes were identified using the GeneMANIA web-tool (<http://genemania.org/>). As illustrated in Fig. 1C and Table 3, the 2 most related transcription factors were identified to be NOTCH1 and YAP1. The YAP1 factor has been widely correlated with asthma [21, 22], and can also influence disease progression by mediating HIF-1 α in both liver cancer and pancreatic cancer [10, 23]. Meanwhile [24], NOTCH1 is involved in the promotion of the GATA3-mediated Th2 response (immunity), which makes NOTCH1 an unlikely candidate responsible for the non-Th2 inflammatory pattern in asthma. As a result, YAP1 (YAP) was selected as the upstream gene for the current study. Moreover, the Multi Experiment Matrix (MEM) website (<https://biit.cs.ut.ee/mem/index.cgi>) predicted that YAP1 was co-expressed with HIF1A (Fig. 1D). Whereas, the hTFtarget website (<http://bioinfo.life.hust.edu.cn/hTFtarget#!/>)

indicated that YAP1 targeted the HIF1A gene (Fig. 1E). Based on the aforementioned *in silico* analysis and previous literatures, we presumed that YAP1 (YAP) may affect the development of asthma through regulation of HIF1A (HIF-1 α), and consequently designed the current study in order to validate our theories.

The results of hematoxylin-eosin staining illustrated that the OVA-induced asthma mice presented with airway remodeling ($p < 0.05$; Fig. 1F) and increased thickening of the airway smooth muscles, airway wall, and airway epithelium mucosa relative to the sham-operated mice ($p < 0.05$; Fig. 1G). These findings verified the successful establishment of OVA-induced murine asthma models. Next, the proportion of Th17 cells was measured in human PBMCs and mouse spleen cells by means of flow cytometry, which revealed an increased proportion of Th17 cells in both asthma patients and mice ($p < 0.05$; Fig. 1H). Meanwhile, detection results of ELISA assay displayed that the serum levels of IL-17A were up-regulated in both asthma patients ($p < 0.05$; Fig. 1I) and asthma mice ($p < 0.05$; Fig. 1J). In addition, RT-qPCR and Western blot analysis determined that ROR γ t mRNA and protein expressions were elevated in PBMCs of asthma patients ($p < 0.05$; Fig. 1K, L) and spleen cells of asthma mice ($p < 0.05$; Fig. 1M, N).

Additionally, results from RT-qPCR and Western blot analysis revealed that YAP and HIF-1 α expressions were both augmented in asthma patients ($p < 0.05$; Fig. 1O, P). Similar trends were observed in YAP and HIF-1 α expressions in OVA-induced asthma mice ($p < 0.05$; Fig. 1Q, R). There is a significant association between asthma and the serum level of HDL-C and thus, HDL-C was employed as a marker to assess the degree of asthma in the current study [20]. As depicted in Fig. 1S, T, HDL-C serum levels were elevated in both asthma patients and mice ($p < 0.05$). Evidence further suggests that HIF-1 α can promote the expression of miR-182 [12]. Meanwhile, the miR-183/96/182 cluster is also known to be significantly up-regulated as a result of Th17 differentiation [13]. Therefore, we speculated that the HIF-1 α signaling pathway might promote Th17 differentiation by regulating miR-182. Subsequently, RT-qPCR was conducted to detect the expressions of miR-96/182/183 in human PBMCs and mouse spleen cells, which showed an upward trend in the miR-96/182/183 expressions in asthma patients and mice, with a more pronounced increase in the miR-182 expression ($p < 0.05$; Fig. 1U, V). Collectively, these results suggested that miR-182, YAP and HIF-1 α were up-regulated, while HDL-C was decreased in asthma.

YAP and HIF-1 α promote the differentiation of CD4⁺ T Cells into Th17 Cells

In order to further verify whether YAP and HIF-1 α were also up-regulated in Th17 cells, we first induced the differentiation of CD4⁺ T cells into Th17 cells *in vitro*. Next, RT-qPCR and Western blot analysis were applied to determine the expressions of YAP and HIF-1 α in the differentiated TH17 cells. As illustrated in Fig. 2A, B, the mRNA and protein expressions of YAP and HIF-1 α were increased in Th17 cells compared to the CD4⁺ T cells ($p < 0.05$).

Additionally, the infection efficiency of YAP/HIF-1 α over-expression or knockdown in the TH17 cells was evaluated and confirmed by means of RT-qPCR and Western blot analysis (Supplementary Fig. 1A, B), wherein sh-YAP-1 and sh-HIF-1 α -1 exhibited superior efficiency and were consequently used for further experimentation ($p < 0.05$). Moreover, Western blot analysis demonstrated that the protein expression of HIF-

1 α was increased in cells with YAP over-expression, while being decreased in YAP knockdown cells ($p < 0.05$; Fig. 2C).

To further investigate whether YAP/HIF-1 α promoted the differentiation of CD4⁺ T cells into Th17 cells, Western blot analysis was applied to detect the protein expressions of YAP and HIF-1 α in Th17 cells with different treatments. As shown in Fig. 2D, cells treated with YAP over-expression or with a combination of YAP over-expression and HIF-1 α silencing exhibited increased YAP protein expressions ($p < 0.05$). However, no significant changes were noted in the YAP protein expression between HIF-1 α over-expression cells and HIF-1 α knockdown cells ($p > 0.05$). After YAP was over-expressed or HIF-1 α was over-expressed, elevated HIF-1 α expressions were found in the Th17 cells ($p < 0.05$). Meanwhile, opposite trends were observed in the Th17 cells following YAP knockdown or/and HIF-1 α knockdown treatment ($p < 0.05$).

After that, the Th17 cell proportion was measured by means of flow cytometry, the results of which (Fig. 2E) displayed that YAP or HIF-1 α over-expression brought about promoted Th17 cell proportion, while YAP or HIF-1 α silencing resulted in reduced Th17 cell proportion ($p < 0.05$). In addition, augmented Th17 cell proportion caused by YAP over-expression was inhibited by HIF-1 α silencing ($p < 0.05$).

Furthermore, the results of ELISA (Fig. 2F), RT-qPCR and Western blot analysis (Fig. 2G, H) revealed that IL-17A levels and ROR γ t expressions were both elevated as a result of YAP or HIF-1 α over-expression, while being reduced in the absence of YAP or HIF-1 α ($p < 0.05$). Additionally, increased IL-17A levels and ROR γ t expressions brought about YAP over-expression were found to be inhibited by HIF-1 α silencing ($p < 0.05$).

All the aforementioned findings highlighted that the differentiation of CD4⁺ T cells into Th17 cells was promoted by over-expressed YAP and HIF-1 α .

HIF-1 α promotes the differentiation of CD4 + T Cells into Th17 Cells by up-regulating miR-182

In the following experiments, we aimed to uncover the underlying mechanism of HIF-1 α in Th17 cell differentiation. RT-qPCR detected that the expression of miR-182 was up-regulated in Th17 cells relative to CD4⁺ T cells ($p < 0.05$; Fig. 3A). In addition, Western blot analysis demonstrated that the protein expression of HIF-1 α was elevated after treatment with HIF-1 α over-expression in HEK293T cells with miR-182-wt or miR-182-mut ($p < 0.05$). Dual luciferase reporter assay revealed that HIF-1 α over-expression brought about a significantly increase in the luciferase activity of miR-182-wt in HEK293T cells ($p < 0.05$; Fig. 3B). Moreover, the ChIP assay illustrated the binding of HIF-1 α to the miR-182 promoter (Fig. 3C): compared to the IgG control, the enrichment of HIF-1 α in the miR-182 promoter was notably increased, and was further promoted after HIF-1 α over-expression ($p < 0.05$). Furthermore, the results of RT-qPCR demonstrated that the expression of miR-182 was increased in cells treated with oe-HIF-1 α or oe-YAP, while being decreased in cells treated with sh-HIF-1 α or sh-YAP ($p < 0.05$; Fig. 3D). These results indicated that HIF-1 α promoted the expression of miR-182 in HEK293T cells.

Additionally, we continued to examine whether YAP/HIF-1 α promoted the differentiation of CD4 + T cells into Th17 cells. The results of RT-qPCR showed a reduction in the miR-182 expression in cells treated with oe-NC + miR-182 inhibitor, oe-HIF-1 α + miR-182 inhibitor or oe-YAP + miR-182 inhibitor, while elevated miR-182 levels

were noted in cells treated with sh-NC + miR-182 mimic, sh-HIF-1 α + miR-182 mimic or sh-YAP + miR-182 mimic ($p < 0.05$). In addition, RT-qPCR and Western blot analysis revealed that the expression of HIF-1 α was augmented in Th17 cells treated with oe-HIF-1 α + miR-182 inhibitor, oe-HIF-1 α + inhibitor-NC, oe-YAP + miR-182 inhibitor or oe-YAP + inhibitor-NC, while being decreased in Th17 cells treated with sh-HIF-1 α + miR-182 mimic, sh-NC + miR-182 mimic, sh-YAP + miR-182 mimic or sh-NC + miR-182 mimic ($p < 0.05$; Supplementary Fig. 2A, B).

The results of flow cytometry further displayed that Th17 cell proportion was reduced in response to treatment with oe-NC + miR-182 inhibitor, oe-HIF-1 α + miR-182 inhibitor, oe-YAP + miR-182 inhibitor, sh-HIF-1 α + mimic-NC or sh-YAP + mimic-NC, but opposite trends were noted upon treatment with sh-NC + miR-182 mimic, oe-HIF-1 α + inhibitor-NC, oe-YAP + inhibitor-NC, sh-HIF-1 α + miR-182 mimic or sh-YAP + miR-182 mimic ($p < 0.05$; Fig. 3E).

Moreover, ELISA demonstrated that IL-17A levels in the supernatant were decreased following treatment with oe-NC + miR-182 inhibitor, which was reversed by sh-NC + miR-182 mimic, oe-HIF-1 α + inhibitor-NC or oe-YAP + inhibitor-NC treatment. In addition, IL-17A levels were found to be diminished in the presence of oe-HIF-1 α + miR-182 inhibitor or oe-YAP + miR-182 inhibitor. Silencing HIF-1 α or YAP brought about decreased IL-17A levels, while treatment with sh-HIF-1 α + miR-182 mimic or sh-YAP + miR-182 mimic abrogated the trend (all $p < 0.05$; Fig. 3F).

Furthermore, the results of RT-qPCR and Western blot analysis illustrated that ROR γ t expressions were diminished in cells upon miR-182 down-regulation or HIF-1 α down-regulation, while being elevated in cells with treated with miR-182 up-regulation or HIF-1 α up-regulation. Meanwhile, dual treatment with oe-HIF-1 α and miR-182 inhibitor inhibited the ROR γ t expression, while treatment with both sh-HIF-1 α and miR-182 mimic brought about increased ROR γ t levels ($p < 0.05$; Fig. 3G, H).

The aforementioned findings highlighted that HIF-1 α up-regulated the miR-182 expression, and consequently promoted differentiation of CD4⁺ T cells into Th17 cells.

miR-182 promotes the differentiation of CD4 + T Cells into Th17 Cells by inhibiting EGR2

In accordance with the cutoff criteria ($|\log FC| > 1$, and $p < 0.05$), 27 DEGs were initially obtained from the GSE64913 dataset (epithelial cells from central airways and from peripheral airways), which included 42 normal samples and 28 asthma samples (Fig. 4A, Supplementary Table 3). Next, 18165 downstream genes of miR-182 were identified from the mirDIP database (score class: medium; <http://ophid.utoronto.ca/mirDIP/>), which were then analyzed with the human transcription factors in the Cistrome database by means of a Venn diagram. Subsequently, EGR2 was found to be a key downstream transcription factor for miR-182 (Fig. 4B, Supplementary Table 4). EGR2 has also been reported to exert an inhibitory role on Th17 cell differentiation [19]. Therefore, we evaluated the expression patterns of EGR2 in human PBMCs (Fig. 4C, D) and mouse spleen cells (Fig. 4E, F) using RT-qPCR and Western blot assays. The results demonstrated that EGR2 expression was down-regulated in asthma ($p < 0.05$). As a result, we speculated that miR-182 might promote Th17 cell differentiation *via* inhibition of EGR2.

Subsequently, we aimed to elucidate and verify the relationship between miR-182 and EGR2. The online website (<http://starbase.sysu.edu.cn/>) predicted the presence of binding sites between miR-182 and EGR2 3'UTR (Fig. 4G). In addition, RT-qPCR analysis revealed that miR-182 expressions were increased in HEK293T cells co-transfected with miR-182 mimic and EGR2-wt or EGR2-mut ($p < 0.05$). Furthermore, dual luciferase reporter assay demonstrated that the luciferase activity of EGR2-wt was reduced ($p < 0.05$), while that of EGR2-mut showed no changes in HEK293T cells following miR-182 mimic transfection ($p > 0.05$; Fig. 4H). The results of RT-qPCR and Western blot analysis shown in Fig. 4I, J showed the diminished expressions of EGR2 in miR-182 mimic-treated cells and elevated EGR2 expressions in miR-182 inhibitor-treated cells ($p < 0.05$). These results indicated that miR-182 targeted EGR2 and inhibited its expression.

After that, we attempted to elaborate whether miR-182 induced Th17 cell differentiation *via* EGR2 inhibition. The results of RT-qPCR and Western blot analysis showed that EGR2 expression was increased upon EGR2 over-expression, while being decreased following sh-EGR2-1 and sh-EGR2-2 treatment ($p < 0.05$; Supplementary Fig. 3A, B). Due to the higher silencing efficiency of EGR2-1 compared to EGR2-2, sh-EGR2-1 was chosen for subsequent experimentation.

Furthermore, RT-qPCR and Western blot analysis (Supplementary Fig. 3C, D) showed that cells treated with oe-EGR2 or both miR-182 mimic and oe-EGR2 presented with elevated expression of EGR2, while those treated with sh-EGR2 exhibited reduced EGR2 ($p < 0.05$). Moreover, cells with miR-182 mimic showed increased miR-182 expression and decreased EGR2 expression ($p < 0.05$). However, compared to cells treated with miR-182 mimic, miR-182 expressions were not notably different from the cells treated with both miR-182 mimic and oe-EGR2 ($p > 0.05$). In addition, cells with miR-182 inhibitor exhibited reduced miR-182 expressions and elevated EGR2 expressions ($p < 0.05$). However, when compared to the cells treated with miR-182 inhibitor, miR-182 expression was not notably different from the cells co-treated with miR-182 inhibitor and sh-EGR2 ($p > 0.05$), while EGR2 was decreased ($p < 0.05$).

In addition, flow cytometry was applied to examine the Th17 cell proportion in treated cells. As illustrated in Fig. 4K, EGR2 over-expression or miR-182 inhibitor inhibited the Th17 cell proportion, while EGR2 knockdown or miR-182 mimic promoted the Th17 cell proportion ($p < 0.05$). When compared with miR-182 mimic treatment alone, the combination treatment of miR-182 mimic and EGR2 over-expression resulted in decreased Th17 cell proportion ($p < 0.05$). Meanwhile, when compared with miR-182 inhibitor treatment alone, the combination treatment of miR-182 inhibitor and EGR2 silencing resulted in increased Th17 cell proportion ($p < 0.05$).

Finally, ELISA was performed to detect IL-17A level ($p < 0.05$; Fig. 4L) and RT-qPCR and Western blot were conducted to determine the ROR γ t expression patterns ($p < 0.05$; Fig. 4M, N). IL-17A levels and ROR γ t expressions were both decreased in cells with miR-182 down-regulation or EGR2 up-regulation, and elevated in cells with miR-182 up-regulation or EGR2 down-regulation ($p < 0.05$). When compared with miR-182 mimic treatment, the combination treatment of miR-182 mimic and EGR2 over-expression led to reduced IL-17A levels and ROR γ t expressions ($p < 0.05$). When compared with miR-182 inhibitor treatment, the combination treatment of miR-182 inhibitor and EGR2 silencing exhibited elevated IL-17A levels and ROR γ t expressions ($p < 0.05$).

Collectively, these findings suggested that miR-182 accelerated the differentiation of CD4⁺ T cells into Th17 cells by inhibiting the expression of EGR2.

EGR2 inhibits the Th17 cell differentiation induced by YAP/HIF-1 α /miR-182

The aforementioned findings suggested that EGR2 over-expression could inhibit miR-182-induced Th17 cell differentiation. In this part of the experiments, we investigated whether EGR2 inhibited the Th17 cell differentiation evoked by the YAP/HIF-1 α axis. RT-qPCR and Western blot analysis (Supplementary Fig. 4A, B) displayed that the YAP expression was increased in CD4⁺ T cells after YAP over-expression alone or both over-expression of YAP and EGR2 ($p < 0.05$). The HIF-1 α expression was also increased after HIF-1 α over-expression alone or both over-expression of HIF-1 α and EGR2 ($p < 0.05$). In addition, cells with both over-expression of YAP and EGR2 exhibited higher EGR2 expressions relative to those with YAP over-expression alone, and the cells with both over-expression of HIF-1 α and EGR2 also exhibited higher EGR2 expressions compared to those with HIF-1 α over-expression alone ($p < 0.05$). Flow cytometry results (Fig. 5A) depicted that YAP or HIF-1 α over-expression promoted Th17 cell proportion, which was inhibited by EGR2 over-expression ($p < 0.05$).

Finally, Fig. 5B illustrates the detection results of IL-17A levels by ELISA and Fig. 5C, D displays the detection results of ROR γ t expression patterns by RT-qPCR and Western blot analysis. IL-17A levels and ROR γ t expressions were both elevated in cells with YAP or HIF-1 α over-expression, while EGR2 over-expression reversed these trends ($p < 0.05$).

These data demonstrated that the differentiation of CD4⁺ T cells into Th17 cells induced by the YAP/HIF-1 α /miR-182 axis might be inhibited by over-expression of EGR2.

Over-expression of EGR2 alleviates asthma and lipid metabolism dysfunction by inhibiting the YAP/HIF-1 α /miR-182 axis in vivo

Next, we sought to verify the aforementioned results *in vivo*. RT-qPCR was performed to detect the miR-182 expression patterns, and RT-qPCR and Western blot analysis were used to determine the protein expression patterns of YAP, HIF-1 α , and EGR2 in mice (Supplementary Fig. 5A, B). Asthma mice were found to present with elevated YAP and HIF-1 α expressions, but reduced EGR2 expressions ($p < 0.05$). Mice following YAP over-expression treatment or dual treatment with YAP over-expression and EGR2 over-expression exhibited increased expressions of YAP ($p < 0.05$). In addition, the treatments with YAP over-expression alone, HIF-1 α over-expression alone, both over-expression of YAP and EGR2, as well as both over-expression of HIF-1 α and EGR2 resulted in increased expressions of miR-182 and HIF-1 α ($p < 0.05$). However, YAP over-expression, HIF-1 α over-expression, or miR-182 over-expression resulted in decreased EGR2 expressions, which could be rescued by EGR2 over-expression ($p < 0.05$). Moreover, when compared with the mice treated with mimic-NC and oe-NC, the expression of miR-182 was still higher in the mice treated with both EGR2 over-expression and miR-182 mimic ($p < 0.05$).

After that, the Th17 cell proportion in mouse spleen was measured by means of flow cytometry. The results are displayed in Fig. 6A, which revealed an increased Th17 cell proportion in the asthma mice and the mice treated with YAP over-expression, HIF-1 α over-expression, or miR-182 over-expression, while being inhibited by EGR2 over-expression ($p < 0.05$). In addition, ELISA was performed to detect the IL-17A levels (Fig. 6B) and RT-qPCR and Western blot were conducted to determine the ROR γ t expression patterns (Fig. 6C, D). IL-17A levels and ROR γ t expressions were elevated in the asthma mice and the mice with YAP over-expression, HIF-1 α over-expression, or miR-182 over-expression, which was observed to be inhibited by EGR2 over-expression ($p < 0.05$).

Th17 cell proportion in mouse lung tissues detected by flow cytometry showed an increase in the asthma mice and the mice with YAP over-expression, HIF-1 α over-expression, or miR-182 over-expression, which was, however, counteracted by the treatment with oe-YAP + oe-EGR2, oe-HIF-1 α + oe-EGR2 or miR-182 mimic + oe-EGR2 ($p < 0.05$; Fig. 6E). Furthermore, hematoxylin-eosin staining results demonstrated significant airway remodeling and increased thickening of the airway smooth muscles, airway wall, and airway epithelium mucosa in the asthma mice and mice with YAP over-expression, HIF-1 α over-expression, or miR-182 over-expression, which was observed to be inhibited by EGR2 over-expression ($p < 0.05$; Fig. 6F, G). In addition, asthma mice treated with oe-YAP, oe-HIF-1 α , or miR-182 mimic presented with reduced HDL-C levels, while increased HDL-C levels were noted following treatment with oe-YAP + oe-EGR2, oe-HIF-1 α + oe-EGR2 or miR-182 mimic + oe-EGR2 ($p < 0.05$; Fig. 6H).

The above data indicated that the over-expression of EGR2 alleviated the exacerbated asthma and lipid metabolism dysfunction evoked by YAP/HIF-1 α /miR-182 signaling *in vivo*.

Discussion

Asthma is a common disease affecting millions of people worldwide characterized by upper airway inflammation and chronic nature [25]. Although asthma can affect people of all ages, a vast majority of asthma patients begin to experience the symptoms in childhood. Therefore, it would be wise to elucidate and control pediatric asthma to avoid any detrimental changes to the long-term quality of life. In the current study, we aimed to investigate the potential molecular mechanism in the development of asthma, and found that the YAP/HIF-1 α factors could potentially augment Th17 cell differentiation, consequently exacerbating asthma and lipid metabolism dysfunction *via* miR-182-mediated EGR2 inhibition.

One of the important findings of the current study is that the factors YAP1 and HIF-1 α were both increased in pediatric asthma. A previous study also noted that YAP was up-regulated in the bronchial airway smooth muscles of chronic asthma mouse models [7]. Meanwhile, YAP can also bind to HIF-1 α in the nucleus and sustain HIF-1 α protein stability in conditions of hypoxic stress in hepatocellular carcinoma cells [10]. Meanwhile, silencing YAP has been documented to markedly down-regulate the protein expression of HIF-1 α , while inhibition of the YAP/HIF-1 α signaling aids in the prevention of angiogenesis of liver sinusoidal endothelial cells [26]. These previous findings in conjunction with our results suggested a positive relationship between YAP and HIF-1 α . Other studies have also illustrated that deficiency of HIF-1 α can reduce eosinophil infiltration, goblet cell hyperplasia, and levels of cytokines IL-4, IL-5, and IL-13 in the lungs of OVA-induced asthma models [11], further highlighting the importance of the elevated levels of HIF-1 α in asthma.

Moreover, HIF-1 α has been reported to facilitate the differentiation of Th17 cells [27], and may serve as an important signaling molecule for the induction of asthma through Th17 cells [14, 15]. HIF-1 α can also cause asthma by means of airway smooth muscle remodeling [28, 29], which is in line with our findings. Whereas, YAP is known to function as an amplifier of the regulatory T cells Treg-reinforcing pathway, holding significant potential as an anticancer immunotherapeutic target [8]. In addition to that, loss of YAP in T cells is known to result in enhanced T-cell activation, differentiation, and function, which translates *in vivo* to an improved ability for T cells to infiltrate and repress the development of tumors [30]. The aforementioned findings and results explained the promoting role of YAP/HIF-1 α in enhancing differentiation of Th17 cells.

Lipid metabolism dysfunction is another potential issue faced by patients plagued by asthma. Accordingly, reductions in good cholesterol levels of HDL-C have been found in asthmatic children [20]. In addition to reduced HDL-C, abnormally high LDL-C and triglycerides are also a common occurrence in pediatric asthma patients [31]. More importantly, the reduction in HDL-C levels has been linked to an increased number of Th17 cells [19]. The significance of this is reflected by the fact that Th17 cells are associated with the production of IL-17, a highly pro-inflammatory cytokine [32]. Moreover, Th17 cells also produce other pro-inflammatory cytokines such as IL-6 and tumor necrosis factor- α , which play trivial role in the inflammatory cascade awakened in the state of asthma [33]. Thus, Th17 cells are rightfully believed to increased immune hyper-responsiveness in an enhanced inflammatory state during asthma [34]. Ni *et al.*, have demonstrated that loss of YAP results in dysfunctional Treg cells failing to inhibit anti-tumor immunity or elicit tumor growth in mice [8]. Additionally, YAP deficiency in T cells enhances T-cell activation, differentiation, and function, as well as improving T-cell responses in cancer [30]. By contrast, the results obtained from the present study revealed that YAP could potentiate Th17 cell differentiation both in PBMCs and asthma mice. This discrepancy may be the difference of the laboratory environment, study subjects and the detection methods used. However, a previous study found significant overexpression and activation of YAP-1 in PBMCs collected from a total of 152 hepatocellular carcinoma cases, and that YAP-1 shares a positive correlation to the percentage of Treg cells; specifically, YAP-1 overexpression in hepatocellular carcinoma T cells induces immunosuppression by promoting Treg cell differentiation [35]. This finding is consistent with the results in this work. It indicates that the role of YAP in Th17 generation could be bilateral, no matter of overexpression or deletion.

Another focus of the current study was microRNAs (miRNAs), which are small non-coding RNA molecules that can modulate gene expression posttranscriptionally by interacting with the 3'-UTR of specific target mRNAs [36]. Herein, we identified that miR-182 could bind to the 3'-UTR of EGR2 and inhibit its expression. Accumulating evidence has shown that EGR2 exerts an inhibitory role on Th17 cell differentiation by negatively regulating Batf [18]. In addition, a previous study found up-regulated expressions of inflammatory transcription factors, such as ROR γ t and Bhlhe40, in EGR2/3 deficient T cells under tolerogenic conditions [37]. Furthermore, EGR2 has the potential to retard the development of chronic rhinosinusitis induced by miR-150-5p in dendritic cells [38]. On the other hand, previous studies have highlighted that miR-182 may promote asthma by stimulating inflammation [39]. Our results also agree with the idea that miRs, like miR-182, miR-30 [40] and miR-221 [41], serve as potential candidates for the treatment of asthma [42]. In line with our findings, miR-182 is also known to be significantly up-regulated upon Th17 differentiation [13]. Therefore, we reasoned that miR-182 allowed Th17 cell differentiation in pediatric asthma by targeting EGR2. Moreover, miR-182 is capable of elevating the HIF-1 α expression. and subsequently promoting breast cancer cell proliferation and

invasion [43], indicating a positive correlation between miR-182 and HIF-1 α . Whereas, HIF-1 α -deficient mice are known to exhibit elevated metabolic rate, hyperventilation, and improved glucose and lipid homeostasis [44]. Hence, based on the aforementioned information, we asserted the hypothesis that amplified EGR2 eliminated Th17 cell differentiation, asthma and lipid metabolism dysfunction driven by YAP/HIF-1 α /miR-182 signaling.

Conclusions

In conclusion, findings in the current study demonstrated that YAP/HIF-1 α enhanced Th17 cell differentiation, consequently exacerbating asthma and lipid metabolism dysfunction *via* miR-182-mediated EGR2 inhibition (Fig. 7). Thus, the YAP/HIF-1 α /miR-182/EGR2 signaling may serve as a novel biomarker for asthma diagnosis and prognosis. However, there are a few notable limitations to our study. First, only OVA was used to stimulate asthma in mice in this study. While human asthma can stem from different causes and exhibit variable forms and severity, animal models can be used to mimic one of more features of the human variation of the disease [45, 46]. Therefore, different animal models should be used to confirm the results uncovered in our study in the future to prevent over-generalization to human situations. Moreover, further studies with different cell lines, different disease models and larger cohorts are essential to validate these findings and expand the translational potential of this direction to realize the full potential of the YAP/HIF-1 α /miR-182/EGR2 signaling axis.

Material And Methods

Ethics Statement

The current study was approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University (approval number: 201908029) and performed in strict accordance with the *Declaration of Helsinki*. Signed consents were obtained from all participants prior to the study. Animal experiments were strictly designed and performed according to the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health, and extensive efforts were made to minimize the suffering of the animals used in the study.

Study Subjects

A total of 29 pediatric patients with asthma (aged < 18 years old; 14 males & 15 females) and 22 healthy volunteers from the First Affiliated Hospital of Nanchang University from June 2018 to December 2018 were enrolled in the current study, and peripheral blood mononuclear cells (PBMCs) were collected in order to extract RNA and protein content.

Ovalbumin (OVA)-Induced Murine Asthma Model

A total of 120 BALB/C mice (aged: 6–8 weeks; weighing 16–20 g) were purchased from the Experimental Animal Center of Nanchang University, amongst which 12 mice were used as normal control, 96 mice were subjected to adenovirus infection, while the remaining 12 mice were used for asthma model establishment. The asthma models were constructed according to a previously reported method [47], with some additional

adjustments. Firstly, the mice were subjected to intraperitoneal injections with 0.2 mL of OVA/aluminum hydroxide on days 0, 7, and 14. After that, starting from day 21, the mice were exposed to 1% OVA inhalation (30 mL) for 30 min once per day, for a total of 7 days. Every time prior to OVA inhalation, each mouse was intraperitoneally injected with 0.2 mL of normal saline. Normal control mice were subjected to matching procedures, with the exception of 30 min of OVA inhalation treatment. Meanwhile, for the mice subjected to adenovirus infection, before administration of OVA and normal saline, the mice were intratracheally injected with phosphate buffer saline (PBS) or adenovirus harboring over-expression plasmids for YAP, EGR2, and HIF-1 α , miR-182 mimic, or the negative control (NC) plasmids (20 μ L, 5×10^{10} pfu/mL) (Fubio Biological Technology Co., Ltd., Shanghai, China) [48]. After model establishment, the mice were anaesthetized with intraperitoneal injections of 3% sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA).

Cell Isolation and Infection

HEK293T cells purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (10569044, Gibco, Langley, OK, USA) supplemented with 10% fetal bovine serum (FBS) (10099141, Gibco), 100 U/mL of penicillin, and 100 U/mL of streptomycin (15070063, Gibco).

Spleen specimens from normal mice were collected and digested using Collagenase D for 30 min at room temperature, followed with treatment using 5 mM of ethylene diamine tetra-acetic acid (EDTA) for 5 min to harvest spleen cells. Next, naive CD4⁺ T cells were isolated from the harvested spleen cells with the help of CD4⁺ CD62L⁺ T cell isolation kits (130-106-643, Miltenyi Biotech, Germany), with a purity of 95%.

Subsequently, the obtained CD4⁺ T cells were seeded in a 6-well plate with Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% FBS at a density of 5×10^5 cells/well. Upon reaching 50–70% confluence, cells were treated with plasmids of miR-182 mimic, miR-182 inhibitor, over-expression and silencing lentiviruses carrying YAP, HIF-1 α , and EGR2, as well as their corresponding NCs (mimic-NC, inhibitor-NC, oe-NC, and sh-NC) purchased from Sangon Biotech (Shanghai, China) for 24 h, followed by 1-week treatment with puro. After that, reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis were performed to measure the efficiency of cell infection. The sequences for mimic and inhibitor are listed in Table 1.

Table 1
Sequences for mimic and inhibitor

Target gene	Sequence (5' – 3')
sh-YAP-1	AGTGCAGCAGAATATGATG
sh-YAP-2	GAGATGGATACAGGTGATA
sh-HIF-1 α -1	F: GATCCCCATCCAGAAGTCACTGGAACCTTTCAAGAGAAGTTCCAGTGACTCTGGATTTTTTGAAA R: TCGAAAAGGTTTTTTTAGGTCTCAGTGACCTTGAAGAGAACTTTCAAGGTCAGTGAACCTACCC
sh-HIF-1 α -2	F: AATTGATGGAACATGATGGTTCACCTTCAAGAGAGTGAACCATCATGTTCCATTTTTT R: CTAGAAAAAATGGAACATGATGGTTCACCTCTTGAAGTGAACCATCATGTTCCATC
miR-182 inhibitor	TTCTACCATTGCCAA'
sh-EGR2-1	F: GATCCATGCGTAACTTCAGTCGTAAGAGAACTTTACGACTGAAGTTACGCATTTTTTTCTCGAGG R: AATTCCTCGAGAAAAAATGCGTAACTTCAGTCGTAAAGTTCTCTTACGACTGAAGTTACGCATG
miR-182 mimic	F: UUUGGCAAUGGUAGAACUCACACU R: UGUGAGUUCUACCAUUGCCAAAUU

In order to differentiate the CD4⁺ T cells into Th17 cells, the infected CD4⁺ T cells were incubated in RPMI-1640 medium containing 10% FBS, 10 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 100 U/mL of penicillin, 100 mg/mL of streptomycin, 50 mg/mL of gentamicin, 1 mM sodium pyruvate, 55 mM 2-ME, 1 mM non-essential amino acids (NEAA), and 2 mM L-glutamine at 37 °C with 5% CO₂ in air for a duration of 72 h. The RPMI medium was prepared with the addition of anti-CD3 (10 µg/mL) (MAB4841), anti-CD28 (1 µg/mL) (MAB4832), transforming growth factor β (TGF- β) (2 ng/mL) (7666-MB), interleukin (IL)-6 (10 ng/mL) (406-ML), anti-IL-4 (10 µg/mL) (MAB404), and anti-IFN- γ (10 µg/mL) (485-MI), which were all purchased from R&D Systems (USA).

Hematoxylin-eosin Staining

Lung tissue sections were fixed with 4% paraformaldehyde at room temperature, and then subjected to hematoxylin-eosin staining (hematoxylin for 60 s and eosin for 3 min) for airway lesion observation. Each section was observed under an optical microscope (BX63, Tokyo, Japan) in a double-blinded manner, followed by measuring the thickness of the airway smooth muscle (µm), airway wall (µm), and airway epithelium mucosa (µm). The experiment was repeated 3 times.

Detection of Th17 Cell Proportion

After 24 h of infection, naive CD4 + T cells were differentiated by the addition of Th17 cell differentiation medium (500 ng/mL phorbol 12-myristate 13-acetate and ionomycin in complete medium) for 72 h. Next, the cells were collected and rinsed once with PBS. Cell suspension of human PBMCs, mouse lung and spleen was then obtained. Incubation was subsequently conducted with fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 antibody at 4°C for 30 min. Thereafter, the cells were further incubated with mouse IL-17/IL-17A PE-conjugated antibody (IC7211P, R&D Systems, USA) at room temperature under dark conditions for 30 min. Finally, the proportion of Th17 cells was detected using a CytoFLEX flow cytometer (Beckman, USA). The experiment was repeated 3 times [49].

RNA Isolation and Analysis

For detection of miR and mRNA expressions, total RNA content was extracted from tissues and cells using the TRIzol reagent (6096020, Thermo Fisher Scientific, USA). After that, the obtained RNA was reverse transcribed into complementary DNA (cDNA) with the use of the TaqMan™ MicroRNA Reverse Transcription Kit (4366596, Thermo Fisher Scientific) for miR-182, and High-Capacity cDNA Reverse Transcription Kit (4368813, Thermo Fisher Scientific) for mRNAs. Then, RT-qPCR was performed with a RT-qPCR kit (11732020, Thermo Fisher Scientific) on a Real-Time PCR system (CFX96, Bio-rad). Finally, the expressions of miR and mRNA were calculated according to the $2^{-\Delta\Delta C_t}$ method, with U6 as the internal reference for miR-182 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for YAP, HIF-1 α , and EGR2. The primer sequences designed by Shanghai Sangon Biotech (Shanghai, China) are presented in Table 2. The experiment was repeated 3 times.

Table 2
Primer sequences for reverse transcription quantitative polymerase chain reaction

Primer	Sequence (5'–3')
hYAP	F: TAGCCCTGCGTAGCCAGTTA
mYAP	R: TCATGCTTAGTCCACTGTCTGT F: CCCTCACCCCTCCCTGAAATCTACAA
	R: AGCATTCCACAGGTCCAAGGCAGA
hHIF-1 α	F: GCTGGCCCCAGCCGCTGGAG R: GAGTGCAGGGTCAGCACTAC
mHIF-1 α	F: AGCTTCTGTTATGAGGCTCACC R: TGACTTGATGTTTCATCGTCCTC
hmiR-182	F: ACACTCCAGCTGGGTTTGGCAATGGTAGAACT R: TGGTGTCGTGGAGTCG
mmiR-182	F: TGCGGTTTGGCAATGGTAGAAC R: TGGTGTCGTGGAGTCG
hEGR2	F: CTTTGACCAGATGAACGGAG R: CCCATGTAAGTGAAGGTCTG
mEGR2	F: CTTGAGCCGAAGTGACCACC R: GCTCTTCCGTTTCCTTCTGCC
hRORyt	F: GCTGTGATCTTGCCCAGAACC R: TGCCCATCATCATTGCTGTTAATCC
mRORyt	F: TGCAAGACTCATCGACAAGG R: AGGGGATTCAACATCAGTGC
hGAPDH	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCAGG
mGAPDH	F: AGGTCGGTGTGAACGGATTTG R: TGTAGACCATGTAGTTGAGGTCA
hU6	F: GTAATACGACTCACTATAGGGAGAAGAG R: CGCGCCTGCAGGTCGAC
mU6	F: GCTTCGGCAGCACATATACTAAAAT R: CGCTTCACGAATTTGCGTGTCAT
Note: h before gene name, Homo sapiens (human); m before gene name, Mus musculus (house mouse).	

Primer	Sequence (5'–3')
hmiR-183	F: GAGGATCCCCGGGTACCAAGGGAGTGGGCAGGCTA R: ATAAGCTTGATATCGTCCCTGCACCCTTGGAAGCA
mmiR-183	F: CGCGCTAT GGCAGTGGTAG R; GTGCAGGGTCC GAGGT
hmiR-96	F: TTC TTC TAG AGG AAG CCC TAA TCC R: TCC CTT CTA GAA AGA TCT ACT CCC C
mmiR-96	F:GCCC GCTTTGGCACTAGCACATT R:CAGTGCAGGGTCC GAGGT
hEGR2	F: CAGGTGTCCACTCCCAGGTCCAAG R: GGCAACTAGAAGGCACAGTCGAGG
Note: h before gene name, Homo sapiens (human); m before gene name, Mus musculus (house mouse).	

Table 3
The degree of genes in
PPI by GeneMANIA
web-tool

Gene	Degree
NOTCH1	36
YAP1	33
H2AFX	15
RUNX1T1	15
USF1	10

Protein Isolation and Analysis

For detection of protein expressions, total protein content was extracted from tissues and cells with a protein extraction kit (78501, Thermo Fisher Scientific), followed by determination of the protein concentration using a bicinchoninic acid kit (23229, Thermo Fisher Scientific). The protein was then electroblotted onto polyvinylidene difluoride membranes (1620177, Bio-Rad, USA), which was then blocked with 5% skim milk or

5% bovine serum albumin (BSA). The membranes were subsequently probed with the following primary antibodies at 4°C overnight: GAPDH (internal control, ab181602, dilution ratio of 1: 5000, Abcam, Cambridge, UK), YAP (ab205270, dilution ratio of 1:1 000, Abcam), HIF-1 α (ab2185, dilution ratio of 1: 1000, Abcam), EGR2 (ab108399, dilution ratio of 1: 1000, Abcam), and ROR γ t (MAB6109, dilution ratio of 1: 1000, R&D Systems). The following day, the membranes were re-probed with horseradish peroxidase-labeled secondary goat anti-rabbit immunoglobulin G (IgG) (ab6721, dilution ratio of 1: 5000, Abcam) or rabbit anti-mouse IgG (ab6728, dilution ratio of 1: 5000, Abcam) for 1 h at room temperature. After that, the membranes were visualized using an enhanced chemiluminescence reagent (1705062, Bio-Rad) and analyzed using Image Quant LAS 4000C (GE Company, USA). The experiment was repeated 3 times.

Dual Luciferase Reporter Assay

The possible binding site between miR-182 and the 3'-untranslated region (3'-UTR) of EGR2 was predicted using an online website (<http://starbase.sysu.edu.cn/>). Then, the synthesized fragments of EGR2-wild type (wt), EGR2-mutant (mut), miR-182-wt, miR-182-mut were introduced into the pGL3 vector (E1751, Promega, USA) using T4 DNA ligase (M0204S, New England Biolabs Inc., MA, USA) and restriction endonuclease. These luciferase reporter plasmids were co-transfected with oe-NC, oe-HIF-1 α , mimic-NC, and miR-182 mimic into HEK293T cells, respectively, and cultured for a duration of 48 h. The luciferase activity was measured using a Dual-Luciferase® Reporter Assay System kit (E1910, Promega Corp., Madison, Wisconsin, USA) on a GLomax 20/20 Luminometer fluorescence detector (E5311, Promega Corp., Madison, Wisconsin, USA). Luminescent signal reflecting the activation of the target reporter gene was compared based on the ratio of the firefly relative light units (RLU) to the Renilla RLU. All vectors were constructed by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China).

HDL-C Determination

The HDL-C content was determined according to the manual of the HDL-C detection kit (ab65390, Abcam). The experiment was repeated 3 times.

Enzyme-linked Immunosorbent Assay (ELISA)

The levels of IL-17A in Th17 cells of mice were measured using an IL-17A ELISA kit (M17AF0, R&D Systems) according to the manufacturer's instructions. The serum levels of IL-17A in human were measured according to the instructions provided by the ELISA kit (D1700, R&D Systems).

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was conducted according to the instructions of the EZ-Magna ChIP kit (EMD Millipore, USA). In brief, the Th17 cells were fixed with 1% paraformaldehyde and cross-linked with glycine for 10 min to produce DNA-protein cross-linking. The cells were then subjected to sonication to shear the DNA into 200–300 bp fragments. The supernatant was then collected and incubated with protein-A coated magnetic beads, followed by the addition of IgG (ab172730, dilution ratio of 1: 100, Abcam) or antibody against HIF-1 α (ab2185, dilution ratio of 1: 20, Abcam). The protein-DNA complexes immobilized by magnetic beads were washed and de-crosslinked. Finally, the miR-182 promoter region in the complexes was determined by RT-qPCR (miR-182 ChIP primer: F: 5'-GAGTGTCCAGGGTTCGTCTG-3', R: 5'-GGTACACTTCTTTGCCCCCA-3').

Statistical Analysis

The Statistic Package for Social Science 21.0 statistical software (IBM Corp, Armonk, NY, USA) was used for statistical analyses. Measurement data were expressed as mean $\pm$ standard deviation. Comparisons between two groups were performed by unpaired *t*-test, and comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. A value of $p < 0.05$ was considered to be statistically significant.

Abbreviations

YAP: Yes-associated protein; miR: microRNA; EGR2: early growth response 2; PBMCs: peripheral blood mononuclear cells; miR-182: microRNA-182; Th17: T-helper 17; HDL-C: high-density lipoprotein cholesterol; PBS: phosphate buffer saline; NC: negative control; EDTA: ethylene diamine tetra-acetic acid; RPMI: Roswell Park Memorial Institute; RT-qPCR: reverse transcription quantitative polymerase chain reaction; NEAA: non-essential amino acids; FITC: fluorescein isothiocyanate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; BSA: bovine serum albumin; 3'-UTR: 3'-untranslated region; RLU: relative light units; ChIP: Chromatin Immunoprecipitation; ANOVA: analysis of variance; GEO: Gene Expression Omnibus; DEGs: expressed genes; PPI: protein-protein interaction; MEM: Multi Experiment Matrix;

Declarations

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Author contributions

Jing Zhou, Ning Zhang and Wei Zhang designed the study. Caiju Lu and Fei Xu collated the data, carried out data analyses and produced the initial draft of the manuscript. Jing Zhou and Ning Zhang contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

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Ethics Statement

The current study was approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University (approval number: 201908029) and performed in strict accordance with the *Declaration of Helsinki*. Signed consents were obtained from all participants prior to the study. Animal experiments were strictly designed and performed according to the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health, and extensive efforts were made to minimize the suffering of the animals used in the study.

Competing interests

The authors declare that they have no conflicts of interest.

Consent for publication

Consent for publication was obtained from the participants.

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Figures

Figure 1

Increased YAP, HIF-1α and miR-182 and decreased HDL-C level in asthma. A, the volcano plot of DEGs related to asthma in peripheral blood cells of asthma patients obtained from the GSE97049 dataset. The red points indicate the significantly upregulated genes, and the green points indicate the significantly downregulated genes; B, the Venn diagram of the DEGs in peripheral blood cells of asthma patients from GSE97049 dataset and the human transcription factors obtained from the Cistrome database; C, the PPI network of the 5 intersected transcription factors in panel B and the related genes; the larger circle at which genes are located reflects higher core degree of the gene and the smaller circle reflects lower core degree. D, the co-expression of YAP1 and HIF1A predicted by the MEM website ($p = 3.76e-18$); E, the target relationship between YAP1 and

HIF1A predicted by the hTFtarget website; F, Hematoxylin-eosin staining of mouse lung tissues (400 ×); G, the diagram depicting the thickness of airway smooth muscle, airway wall, and airway epithelium mucosa of mice; H, Th17 cell proportion in human PBMCs and mouse spleen cells detected by flow cytometry; I, the serum level of IL-17A in asthma patients measured by ELISA; J, the serum level of IL-17A in asthma mice measured by ELISA; K, RORyt mRNA expression in human PBMCs detected by RT-qPCR; L, Western blot analysis of RORyt protein in human PBMCs; M, RORyt mRNA expression in mouse spleen cells detected by RT-qPCR; N, Western blot analysis of RORyt protein in mouse spleen cells; O, mRNA expression of YAP and HIF-1α in human PBMCs detected by RT-qPCR; P, Western blot analysis of YAP and HIF-1α proteins in human PBMCs; Q, mRNA expression of YAP and HIF-1α in mouse spleen cells detected by RT-qPCR; R, Western blot analysis of YAP and HIF-1α proteins in mouse spleen cells. S, the serum level of HDL-C in asthma patients; T, the serum level of HDL-C in asthma mice; U, miR-183/96/182 expression in human PBMCs detected by RT-qPCR; V, miR-183/96/182 expression in mouse spleen cells detected by RT-qPCR. Comparisons between two groups were conducted by unpaired t test; * $p < 0.05$, compared with the normal individuals (normal) or normal mice (normal-M); $n = 22$ in normal individuals; $n = 29$ in asthma patients; $n = 12$ in normal mice; $n = 12$ in asthma mice.

Figure 2

YAP and HIF-1α promoted the differentiation of CD4⁺ T cells into Th17 cells. A, mRNA expression of YAP and HIF-1α in Th17 cells detected by RT-qPCR; B, Western blot analysis of YAP and HIF-1α proteins in Th17 cells; C, Western blot analysis of HIF-1α protein in Th17 cells after YAP overexpression or knockdown; D, Western blot analysis of YAP and HIF-1α proteins in Th17 cells following different treatments; E, Th17 cell proportion after different treatments detected by flow cytometry; F, IL-17A serum level in cell supernatant after different treatments by ELISA; G, RORyt mRNA expression in cells after different treatments detected by RT-qPCR; H, Western blot analysis of RORyt protein in cells after different treatments. Comparisons between two groups were conducted by unpaired t test, and those among multiple groups were conducted by one-way ANOVA with Tukey's post hoc test; the experiments were repeated 3 times; * $p < 0.05$, compared with the CD4⁺ T cells, or cells treated with sh-NC, oe-NC, or sh-NC + oe-NC; # $p < 0.05$, compared with the cells treated with oe-YAP + sh-NC.

Figure 3

HIF-1 α upregulated miR-182 and then promoted Th17 cell differentiation. A, miR-182 expression in Th17 cells detected by RT-qPCR; B, the HIF-1 α protein expression by Western blot analysis and the effect of HIF-1 α on the miR-182 promoter activity detected by dual luciferase reporter gene assay; C, the binding of HIF-1 α to miR-182 promoter detected by ChIP assay; D, miR-182 expression after HIF-1 α /YAP overexpression or knockdown detected by RT-qPCR; E, Th17 cell proportion after different treatments detected by flow cytometry; F, the level of IL-17A in cell supernatant after different treatments detected by ELISA; G, ROR γ t mRNA expression in cells after different treatments detected by RT-qPCR; H, Western blot analysis of ROR γ t protein in cells after different treatments. Comparisons between two groups were conducted by unpaired t test, and those among multiple groups were conducted by one-way ANOVA with Tukey's post hoc test; the experiments were repeated 3 times; * $p < 0.05$, compared with the normal individuals (normal), normal mice (normal-M), CD4 $^{+}$ T cells, cells treated with sh-NC, oe-NC, oe-NC + inhibitor-NC, or sh-NC + mimic-NC; # $p < 0.05$, compared with the cells treated with oe-HIF-1 α + inhibitor-NC or sh-HIF-1 α + mimic-NC; & $p < 0.05$, compared with the cells treated with oe-YAP + inhibitor-NC or sh-YAP + mimic-NC. $n = 22$ in normal individuals; $n = 29$ in asthma patients; $n = 12$ in normal mice; $n = 12$ in asthma mice.

Figure 4

miR-182 inhibited EGR2 expression and then promoted Th17 cell differentiation. A, the heatmap of DEGs related to asthma in respiratory epithelial cells obtained from the GSE64913 dataset; the right upper histogram represents color gradation; B, the Venn diagram of the DEGs in respiratory epithelial cells from the GSE64913 dataset, downstream genes of miR-182 from the miRDIP database and human transcription factors from the Cistrome database; C, EGR2 mRNA expression in human PBMCs detected by RT-qPCR; D, Western blot analysis of EGR protein in human PBMCs; E, EGR2 mRNA expression in mouse spleen cells detected by RT-qPCR; F, Western blot analysis of EGR2 protein in mouse spleen cells; G, the binding site between miR-182 and EGR2 3'UTR in mice predicted by the starbase website; H, miR-182 expression detected by RT-qPCR and the binding of miR-182 to EGR2 confirmed by dual luciferase reporter assay; I, miR-182 expression and EGR2 mRNA expression detected by RT-qPCR; J, Western blot analysis of the EGR2 protein in cells after different treatments; K, the Th17 cell proportion after different treatments determined by flow cytometry; L, the level of IL-17A after different treatments by ELISA; M, RORγt mRNA expression in cells after different treatments detected by RT-qPCR; N, Western blot analysis of the RORγt protein in cells after different treatments. Comparisons between two groups were conducted by unpaired t test, and those among multiple groups were conducted by one-way ANOVA with Tukey's post hoc test; the experiments were repeated 3 times; * $p < 0.05$, compared with the normal individuals (normal), normal mice (normal-M), cells treated with mimic-

NC, inhibitor-NC, oe-NC + mimic-NC, or sh-NC + inhibitor-NC; # $p < 0.05$, compared with the cells treated with miR-182 mimic + oe-NC or miR-182 inhibitor + sh-NC; $n = 22$ in normal individuals; $n = 29$ in asthma patients; $n = 12$ in normal mice; $n = 12$ in asthma mice.

Figure 5

Overexpression of EGR2 inhibited Th17 cell differentiation induced by the YAP/HIF-1α/miR-182 signaling. A, Th17 cell proportion after different treatments determined by flow cytometry; B, the level of IL-17A in cell supernatant after different treatments measured by ELISA; C, RORγt mRNA expression in cells after different treatments detected by RT-qPCR; D, Western blot analysis of RORγt protein in cells after different treatments. Comparisons among multiple groups were conducted by one-way ANOVA with Tukey's post hoc test; the experiments were repeated 3 times; * $p < 0.05$, compared with the cells treated with oe-NC; # $p < 0.05$, compared with the cells treated with oe-YAP or oe-HIF-1α.

Figure 6

EGR2 overexpression alleviated the asthma and lipid metabolism dysfunction induced by the YAP/HIF-1 α /miR-182 axis in vivo. A, the Th17 cell proportion in mouse spleen cells determined by flow cytometry; B, the level of IL-17A in mouse serum measured by ELISA; C, ROR γ t mRNA expression in mouse spleen cells detected by RT-qPCR; D, Western blot analysis of ROR γ t protein in mouse spleen cells; E, Th17 cell proportion in mouse lung tissues detected by flow cytometry. F, hematoxylin-eosin staining of mouse lung tissues (400 $\times$); G, the thickness of airway smooth muscle, airway wall, and airway epithelium mucosa of mice; H, the level of HDL-C in mouse serum. Comparisons among multiple groups were conducted by one-way ANOVA with Tukey's post hoc test, $n = 12$ for mice following each treatment; * $p < 0.05$, compared with the mice treated with sh-NC, oe-NC, or mimic-NC + oe-NC; # $p < 0.05$, compared with the mice with oe-YAP, oe-HIF-1 α , or miR-182 mimic + oe-NC.

Figure 7

The mechanistic diagram illustrating the role of the YAP/HIF-1α/miR-182/EGR2 axis in asthma. The overexpression of YAP and HIF-1α promoted the expression of miR-182, thereby inhibiting EGR2 expression, increasing the expression of RORγt and IL-17A, and prompting the differentiation of CD4⁺T cells into Th17 cells, which ultimately aggravated asthma and lipid metabolism dysfunction.

Supplementary Files

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