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Effects of THC on human amniotic epithelial cell proliferation and migration.

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Abstract

Background: The deleterious effects of cannabis consumption for fertility and pregnancy outcome are recognized for years. The main psychoactive molecule of cannabis, Δ(9)-tetrahydrocannabinol (THC) is able to cross the placenta barrier and cause alterations in fetal growth, low birth weight and preterm labor. However, the effects of THC on the human placenta amnion are still unknown.

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Methods: The distributions of CB1R and CB2R in human amnion tissues were observed by immunohistochemistry (IHC). Human amniotic epithelial cell proliferation and migration in response to THC treatment were measured by MTS and transwell assays, respectively. The PCR array was performed to study the key regulators involved in the cell migration. The protein levels of CB1R、CB2R in amnion tissues and MMP2、MMP9 in cells were detected by western blotting. Small interfering RNAs (siRNAs) were used to knockdown MMP2 and MMP9 in WISH cells.

Results: Our results indicated that both CB1R and CB2R primarily identified in the epithelial layer of human placental amnion tissue. The CB1R expression in the amnion tissue was higher in the preterm group than normal control. High-dose of THC (30uM, but not 20 and 10uM) significantly inhibited ($p<0.01$) human amniotic epithelial cell lines (WISH) proliferation. Meanwhile, THC at both 10uM and 20uM ($p<0.05$) significantly suppressed cells migration in both WISH and primary human amniotic epithelial cells. The PCR array data and siRNA experiments demonstrated that MMP2/9 were tightly involved in the regulation of THC-inhibited cell migration in WISH cells.

Conclusion: These results suggested that THC inhibited the migration of human amniotic epithelial cell through the regulation of MMP2 and MMP9, which in turn altered the development of the amnion during the gestation and partially resulted in preterm labor and other adverse pregnancy outcomes.

Key words: Δ(9)-tetrahydrocannabinol (THC); amniotic epithelial cell; cannabinoid receptor type 1 (CB1); proliferation; migration; MMPs

Introduction:

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Cannabis is the most consumed illegal drug around the world. Its consumption during pregnancy is associated with many gestational complications, such as FGR (fetal growth restriction), low birth weight and preterm labor. The major psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), due to its lipophilicity, is able to cross the placental barrier and accumulate in maternal milk. In this way, the consumption of cannabis during gestation and lactation is implicated in deficient fetal growth, low birth weight and preterm labor (Fergusson et al. 2002; Jaques et al. 2014). Increasing use of cannabinoids (marijuana) during the last decades has raised questions about their effects on the mother and developing fetus.

CB1 (cannabinoid receptor 1) and CB2 (cannabinoid receptor 2) are the two main molecular targets of the major psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC). In recent studies, CB1 and CB2 were found expressed in several organs and tissues and participated in several cell events such as proliferation, migration, apoptosis and inflammation (Costa et al. 2015; Fonseca et al. 2013). Expression of cannabinoid CB1 and CB2 receptors' messenger RNA (mRNA) has been detected as early as the preimplantation period in the embryonal mouse and human term placenta(Paria and Dey 2000; Park et al. 2003), additionally, CB1 receptor mRNA has been found in the uterus, and the crucial role of this receptor in the normal onset of labor has been also suggested(Das et al. 1995; Wang et al. 2008).

Preterm labor, a pregnancy-specific disease defined by deliveries occurred at less than 37 weeks' gestational age, remains to be one of the major causes of maternal and fetal morbidity and mortality, affecting 5-9% of all pregnant women(Goldenberg et al. 2008). There are many maternal or fetal characteristics that have been associated with preterm birth including the impaired amnion structure and function. It is reported that amnions usually rupture during the process of labor, which is associated with significant fetal and

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maternal morbidity and mortality. Rupture of fetal membranes can initiate parturition at both term and preterm labor. The amnion epithelium plays an important role in structural remodeling of the membranes preceding rupture of fetal membranes.

The aims of this study were to investigate the expression of the CB1 and CB2 receptors in human amnion from preterm and term pregnancy, to evaluate the direct effects of the Δ^9-THC on human amnion during pregnancy, and to determine whether the amniotic effects of the Δ^9-THC was mediated by the stimulation of the CB1 or CB2 receptors and the underlying molecular mechanism.

Materials and Methods

Patients and samples collection

Placental tissues were obtained from a portion of normal (39 ± 0.47 wks, n = 10) and preterm labor patients (33 ± 1.0 wks, n = 11) and obtained immediately (< 30 mins) after delivery by caesarean section. Small pieces (~ $0.5cm^3$) were cut from the fetal part of the placentas under the aseptic conditions and washed briefly in sterile PBS to remove maternal blood contamination. All samples were frozen within 15 minutes of delivery and stored in liquid nitrogen for Western blot analysis. Additional placental tissues were fixed at 4°C using 4% paraformaldehyde in 10 mM PBS within 24 hours and embedded them in paraffin for immunohistochemistry (IHC).

Preterm is defined as a delivery before 37 weeks of gestation. All the preterm patients exhibited spontaneous preterm labor and/or premature rupture of membranes without evidence of infection

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(microbiological culture investigations and histopathological examinations), proteinuria, hypertensive disease or maternal co-morbidities (Goldenberg et al. 2008).

The Scientific and Ethical Committee of the Shanghai First Maternity approved the collection of placental samples and Infant Hospital affiliated with Tongji University. All of the samples were collected with a written informed consent provided by the participants.

IHC

Immunolocalization of CB1 and CB2 in the placental tissues was visualized by indirect detection via the avidin-biotinylated peroxidase complex method (Vector Laboratories, Burlingame, CA) as described (Jiang et al. 2010). Tissue sections (5 μm) were deparaffinized and dehydrated. Endogenous peroxidase activity was quenched by immersing the tissue sections in 3% H_2O_2 in methanol for 10 min. After blocking the non-specific binding with 1% horse serum albumin for 20 min, the tissue sections were probed with a rabbit anti-CB1 antibody (1:50, Abcam, ab3558, Cambridge, UK) and a rabbit anti-CB2 antibody (1:50, Abgent, AP10674b, San Diego, USA).

Western blot analysis

Total placental tissue lysates were prepared by homogenization as previously described (Chung et al. 2004; Jiang et al. 2010). The extracted protein concentration was measured with BCA Protein Assay Kit (Thermo Scientific). 20 μg proteins were separated on 10% SDS-PAGE gels. Proteins were transferred to polyvinylidenedifluoride membranes (Roche Diagnostics, No. IPVH00010, Indianapolis, IN). After blocking (5% milk in TBST) in room temperature for 1 h, the membranes were probed with a rabbit anti-CB1 antibody (1:1000, Abgent, AP19620c, San Diego, USA) and a rabbit anti-CB2 antibody (1:50, Abgent, AP10674b, San Diego, USA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10000, Abmart, Shanghai,

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China) as an endogenous loading control, respectively. Proteins were visualized using enhanced chemiluminescence reagents (Thermo Scientific, No. KC-5G4) and the immunoreactive signals were analyzed by densitometry using the Image-J imaging analysis software (NIH, Bethesda, MD).

Cell Proliferation and Migration

To explore the role of THC in human amniotic epithelial cells, cell proliferation and migration were conducted using human amniotic epithelial (WISH) cell line, originally purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The WISH cell, an immortalized cell line, is widely used as an amniotic epithelial cell model for studying normal amniotic epithelium function. WISH cells were cultured in MEM (MEM, KeyGEN, KGM41500N, Jiangsu, China) supplemented with 10% fetal bovine serum (FBS; Gibco, No. 10099) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Gibco, No. 15140). All cells were incubated at 37ºC in a humidified atmosphere containing 5% CO2.

For the cell proliferation assay, cells seeded in 96-well plates (2000 cells/well) were cultured in MEM media. After 16h, attached cells were treated with different concentrations of THC (0-30 uM; Sigma-Aldrich, T-005, USA) in media supplemented with 10% FBS and 1% P/S under nomoxia (20% oxygen) for up to 48 hours. The number of cells was determined using the MTT (Promega, G3580, WI, USA) method(Sultan et al. 2015). Wells containing known cell numbers (0, 625, 1250, 2500, 5000, 10,000, 20,000 or 40,000 cells/well; 6 wells/cell density) were treated in the similar fashion to establish standard curves. Four independent experiments were run for the cell proliferation assay.

Commented [菊蕾1]: normoxia

Cell migration was evaluated using a 24-Multiwell BD Falcon FluoroBlok Insert System (8.0 μm pores; BD Biosciences, San Jose, CA). After treatment with 0、10 and 20 uM THC for 32hr, cells (40,000 cells/well) were seeded into the insert (topside of membrane) in the MEM media with 1% FBS. The bottom wells of the

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chamber were filled with MEM media with 10% FBS. After 16 h treatment with 0、10 and 20 uM THC under normoxic condition, cells that migrated to the bottom of the inserts were stained with calcein AM (0.2 μg/ml; Invitrogen, No.C3100MP) for 30 min, examined and recorded by an inverted microscope mounted with a CCD camera. The numbers of migrated cells were counted using the Image-J imaging analysis software (NIH, Bethesda, MD).

CB1R inhibitor

CB1R inhibitor SR141716 (Rimanonbant) was from Selleckchem (Houston, TX, USA). It was initially dissolved in DMSO to a concentration of 10mM and stored at -80℃. WISH cells were pre-treated with 1uM SR141716 before it exposed to THC.

Amnion epithelial cell preparation

Fetal membranes were collected under a protocol approved by the Scientific and Ethical Committee of the Shanghai First Maternity and Infant Hospital affiliated with Tongji University. Amnion was peeled off the chorion and washed three times in cold PBS (pH 7.5). For amnion epithelial cell preparation, amnion tissue was digested with 0.125% trypsin (Sigma, St.Louis, MO) and 0.02% DNAase (Sigma) twice for 20 min at 37 ^{0}C. The digestion media were collected, and the remaining amnion tissue was washed vigorously with PBS three times to wash residual epithelial cells off the amnion tissue. The wash solution was then combined with the previous trypsin digestion media. The digestion medium was then collected. The trypsin (epithelium) media was centrifuged at 2300 rpm for 10 min. Cell pellets were collected and resuspended in DMEM without phenolred (Sigma). Resuspended cells were loaded onto pre-prepared discontinuous Percoll (Sigma) gradients (5, 20, 40, and 60%, respectively), and the gradients were centrifuged at 2500 rpm for 20 min. A single band of cells around 20% Percoll concentration was collected and diluted with DMEM containing 10% fetal bovine

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serum (FBS; Gibco, No. 10099) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Gibco, No. 15140) to a density of 10^6 cells/ml(Sun et al. 2003). Cells ($3*10^6$) were plated in each well of a 6-well plate. Cell culture was maintained at 37℃with a water saturated atmosphere of 5% CO_2 in air.

Immunocytochemical staining for cytokeratin

To identify the cell type that was obtained after trypsin digestion, immunocytochemical staining for cytokeratin (epithelial cell marker) was carried out on cells cultured for 3d on chamber slides using the avidin biotinperoxidase method (Vector ABC, Vector Laboratories, Burlingame, CA) (Blumenstein et al. 2000). The cells were washed with PBS and fixed with 4% paraformaldehyde. Before applying primary antibodies, endogenous peroxidase activity was quenched in 0.3% H_2O_2, and then the cells were incubated with normal blocking serum. After removal ofexcess serum, the monoclonal cytokeratin antibodies (Sigma) at 1:1000 dilution were applied respectively as primary antibodies. After incubation with the primary antibodies for 30 min at 37 C, the cells were washed and appropriate secondary antibodies were then applied. Incubation was further carried out for 30 min at 37℃. After the cells were washed, cells were incubated with Vectastain ABC reagent (Vector) for 30 min. The color reactions were developed using 3-amino-9-ethyl carbazole (redcolor). Cells were counterstained with Carazzi's hematoxylin and examined by light microscopy.

PCR array

Total RNA from each sample was used for reverse transcription with an RT-PCR Kit (catalog#CTB101; CT biosciences, China) on an ABI 9700 thermocycler (ABI, Foster City, CA). For reverse transcription, 4 uL of total RNA was mixed with 10 uL of OligodT Primer (10 uM), and the solution was incubated at 70^0 C for 10 min and then quickly cooled on ice for 2 min. The cooled solution was mixed with 4uL of 5 × reverse transcription buffer, 1 uL of dNTP (10 mM), 0.5 uL of RNasin (40 U/uL), and 0.5 uL of reverse transcriptase

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(200 U/uL). Reverse transcription was performed at 42^0C for 1 hour, followed by an inactivation reaction at 70^0C for 15 min. The resulting cDNA was stored at -20^0C until used. PCR arrays were performed with customized PCR containing predispensed primers (CT biosciences, China) on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using SYBR Master Mix (catalog#CTB101; CT biosciences, China). The thermocycler parameters consisted of an initial denaturation at 95^0C for 2 min followed by 45 cycles of denaturation at 95^0C for 10 s and annealing at 60^0C for 20 s. Relative changes in gene expression were calculated using the ΔΔCt (threshold cycle) method. The housekeeping genes (Bactin and GAPDH) were used to normalize the amount of RNA. Fold change values were calculated using the 2-ΔΔCt formula(Bin Yu 2015).

RNA isolation, reverse transcription and qRT-PCR

Total RNA of WISH cells treated with 20 uM THC were extracted using Trizol (Invitrogen, Carlsbad, USA). The concentration of RNA was measured by a spectrophotometer (ND 2000, Nanodrop Inc, Wilmington, Del). One micrograms of RNA was used for reverse transcription. RNA was reverse-transcribed using SuperScript First Strand cDNA System (Takara, Japan) according to the manufacturer's instructions. MMP2, MMP9 and GAPDH genes were amplified in a fluorescence reader ABI Stepone system (Applied Biosystems, Foster City, CA, USA). The amplification was carried out using SuperReal PreMix Plus (SYBR Green) (TIANGEN, China) according to the manufacturer's instructions. Cycling conditions were as follows: initial enzyme activation at 95°C for 20 s, followed by 40 cycles of denature at 95°C for 3 s, anneal/extend at 60°C for 30 s. The primer sequences for MMP2 gene were: 5'-TACAGGATCATTGGCTACACACC-3' (forward) and 5'-GGTCACATCGCTCCAGACT-3' (reverse); the primer sequences for MMP9 gene were 5'-TGTACCGCTATGGTTACACTCG-3' (forward) and 5'-GGCAGGGACAGTTGCTTCT-3' (reverse); and the

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primer sequences for GAPDH gene were: 5'-TGGGCTACACTGAGCACCAG-3' (forward) and 5'-AAGTGGTCGTTGAGGGCAAT-3' (reverse). The relative expression of the mRNA was calculated with the following formula: Ratio = 2-ΔCt, in which ΔCt = (Ct target gene - Ct β-actin). Each experiment was carried out in triplicate.

Cell Transfections

Transfections were performed using the HiPerfect reagent (QIAGEN, German) according to the manufacturer's guidelines. Transfection conditions for WISH cell line were first optimized to result in 20%-30% transfection efficiency with a cell viability of more than 80%. The transfection efficiency used was 9.8% ± 0.8%. On day 1, specific siRNAs were transfected at a concentration of 100nM using the transfection reagents following the manufacturer's protocol. The transfection medium was then replaced daily with regular culture media containing serum for at least 48 hours prior to study. All studies were performed in quadruplicate unless otherwise specified.

Statistical Analysis.

All data are expressed as mean ± SEM. The differences between groups were analyzed by Student's two tailed *t*-test when two groups were analyzed or ANOVA if more than two groups were analyzed. A P value <0.05 was used to indicate statistically significant differences

Results

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1. CB1 and CB2 protein were present in the human amniotic epithelial cells in amnion. CB1 expression was increased in preterm labor than term labor.

The clinical characteristics from full term and preterm labor patients are shown in Table 1. No significant discrepancies were observed in the maternal age among the groups studied. Consistent with previous epidemiologic studies, patients with preterm labor had smaller ($p \leq 0.001$) gestational ages and lower fetal weights ($p \leq 0.001$) compared to the full term. IHC was performed to localize CB1/CB2 in the human amniotic epithelial cells in amnion. Positive brownish staining for CB1/CB2 was observed primarily in human amniotic epithelial cells in the full term pregnancy (Fig. 1A).

Total amniotic CB1/CB2 protein levels in amnion during pregnancy were investigated by Western blot analysis. We observed a significant elevation in CB1 in the preterm pregnancies as compared with full term pregnancies (Fig.1B). CB2 showed no remarkable differences between the two groups (Fig.1B).

We supported this data by Immunohistochemical localization of CB1 and CB2 in full term labor and preterm labor amnion (Figure 1C). In Fig.1C, we observed the same elevation of CB1 in the preterm labor when compared with term labor. The CB2 showed no change in the two groups.

2. THC inhibited cell proliferation and migration in WISH.

In the present study, we found the CB1 and CB2 protein presented in the amnion tissue. Then, we wanted to examine the role of THC in regulating the amniotic epithelial cell function. We determined the effects of THC on amniotic epithelial cell proliferation and migration using WISH cells (Fig.2). We found that THC at 30uM concentration significantly suppressed WISH cells proliferation, but no such effect was observed under THC

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at 10uM and 20uM concentration. In addition, THC (10uM) ($p<0.05$) suppressed cells migration, and THC (20uM) suppressed cells migration completely.

3. THC inhibited cell migration through CB1 in WISH.

The selective CB1 antagonist SR 141716 was used to testify the role of CB1 in THC-suppressed migration. Our data showed that the selective CB1 antagonist partially rescued the THC-suppressed cell migration. (Fig.3)

4. THC inhibited cell migration in human primary amniotic epithelial cells derived from both term labor and preterm labor.

Besides WISH cell line, we also testified the effect of THC on human primary amniotic epithelial cells (hAECs). After we isolated the cells from amnions of normal pregnancy labors, we identified the cells with immunofluorescence. Cytokeratin, the marker of hAECs, showed positive in the human primary amniotic epithelial cells (Fig.3A). The effect of THC in primary human amniotic epithelial cells (hAECs) migration was suppression in accordance with WISH cells. THC inhibited hAECs migration significantly at 10uM and 20μM (Fig.3B 3C).

Seeing the CB1 receptor was higher in preterm amnion tissues and CB1 receptor may participate in the THC-suppressed cell migration, we isolated the primary amniotic epithelial cells from preterm labors. In Fig.3B, we can find that the cell migration ability of preterm labors was decreased when compared with term labors. THC inhibited the preterm labor derived cells notably at 10uM and 20μM consistent with the data in WISH cell line and normal term labor derived primary cells. The inhibition of THC on cell migration was slightly increased in preterm cells. At 10uM THC, the fold change of migrated cell numbers was 1.27 fold

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higher in term cells compared with preterm cells (0.3113±0.06511 versus 0.2454±0.04918 for term and preterm cells, P=0.4642). At 20uM THC, the fold change of migrated cell numbers was 10.5 fold higher in term cells compared with preterm cells (0.07218±0.02407 versus 0.006873±0.006873 for term and preterm cells, P=0.0595).

5. THC inhibited the expression of MMP2 and MMP9 in WISH cells.

To find the key gene in THC-suppressed cell migration, we performed a PCR-array. In the result, MMP9 was found to have a decline in the expression (Fig 5A). It is well known that MMP2 is also a key regulator on the cell migration in the MMP family. We check the total MMP2/9 protein levels after WISH cells treated with 20uM THC by PCR analysis and Western blot analysis. In figure 5B, total MMP2/9 protein levels were found to have a decline.

6. MMP2 and MMP9 involved in WISH cell migration.

In order to investigate the role of MMP2 and MMP9 in WISH cell migration, we use siRNA to knockdown the expression of MMP2 and MMP9 in WISH cells. The knockdown effects on MMP2 and MMP9 transcription and expression were confirmed by PCR (Fig.6A) and Western Blot (Fig.6B). Moreover, by silencing MMP2 and MMP9, the in vitro experiments showed WISH cells revealed a decreased cell migration ability when compared with NC transfected cells (Fig.5C 5D).

Discussion

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The hazards of cannabis consumption during the gestational period for pregnancy outcome and long-term child development are well recognized. However, the impact of the major psychoactive compound of cannabis, THC, in the feto-placental unit has not been explored. In the present work, THC effects on human amniotic epithelial cells were investigated. We reported that CB1 and CB2 protein were present in the human amniotic epithelial cells in amnion. CB1 expression was increased in preterm labor than term labor. THC inhibited cell migration through CB1 receptor in WISH cells. Moreover, THC inhibited the expression of MMP2 and MMP9 which probably involved in THC-induced suppressed cell migration.

In this study, we demonstrated that CB1 and CB2 protein were present in amnion. Previously CB1 mRNA has been detected in the central nervous system and in peripheral tissues that include heart, lung, bladder, and adrenal gland (Izzo et al. 2001; Pertwee 1997). Interestingly, we found that CB1 expression was increased in preterm labor than term labor while CB2 showed no difference. Little is known about the physiologic role of the CB2 receptors, but CB2 mRNA is present mainly in immune cells with particularly high levels in B cells and natural killer cells(Galiegue et al. 1995), which plays important role in immune regulation. Maria et al(Bariani et al. 2015) reported that in their model of LPS-induced preterm labor, they observed an increased in CB1 receptor protein levels in uteri of pregnant mice and no changes in CB2 receptor expression at protein. Our study suggested that CB1 but not CB2 in amnion tissues appeared to have a major role in the preterm labor that is elicited by cannabinoids.

Importantly, we found that THC at 10uM ($p<0.05$) suppressed cell migration, and at 20uM suppressed cells migration completely in WISH cells. Interestingly, the selective CB1 antagonist SR 141716 partially rescued the THC-suppressed cell migration in WISH cells, suggested that THC participated in the amniotic epithelial cell migration through CB1 receptor. It is noteworthy that migration event of amniotic epithelial

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cells plays a key role in the onset of normal and preterm labor. The impaired migration function may result in premature of fetal membranes. Based on above findings we speculated that the increased CB1 levels in preterm amnion may enhanced THC-inhibited cell migration, which in turn induced dysfunction of human amniotic epithelial cells.

Previously, we have established that THC inhibited WISH migration at 10uM and 20uM, which was later tested same in primary cells whether derived from term labors or preterm labors. To our knowledge, this is the first study to characterize the changes in the THC-treated cell migration ability of primary amniotic epithelial cells. The fact that the cell migration ability is decreased in preterm labor derived cells may afford new ideas for the onset of preterm labor. Moreover, after treatment with THC, the inhibition of cell migration was slightly increased in preterm cells, especially at 20uM THC. These data are consistent with the hypothesis that the increased CB1 levels in preterm amnion may enhance THC-inhibited cell migration.

It is well established that MMP2 and MMP9 play important roles in migration and invasion. MMP2 and MMP9 appeared to be necessary for enhanced migration and invasion of lung cancer cells, hepatocellular carcinoma cells and human pulmonary artery smooth muscle cells(Spiekerkoetter et al. 2009; Sze et al. 2011; Zhan et al. 2014).Until now, little has been known about the role of MMP2 and MMP9 on the human amniotic epithelial cell migration. In our study, the PCR array data showed the expression of MMP family was decreased. By silencing MMP2 and MMP9, our in vitro experiments showed WISH cells revealed decreased cell migration ability. Our current study demonstrated that MMP2 and MMP9 played an important role in THC-induced suppressed cell migration.

In conclusion, we observed that CB1 protein levels increased significantly in the preterm labor when compared with full term labor. Moreover, THC inhibited cell migration through CB1 receptor in WISH cells,

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in which MMP2 and MMP9 probably played an important role. To date, there are questions around THC/CB1/MMP2/MMP9 signaling in the occurrence of preterm labor. Thus, better understanding of THC-related mechanisms would help us to visualize the development of pregnancy-related disorders such as preterm labor, in order to explore effective strategies.

Disclosure Statement

The authors declare that they have no competing interests.

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Figure legend:

Fig.1 Immunohistochemical localization of CB1 and CB2 in full term labor amnion (A). Brownish color indicates positive staining for CB1 and CB2. Western blot analysis for CB1 and CB2 protein in human amnion from normal and preterm pregnancies (B, C). Amnions were obtained from the term labor (n = 10) and preterm labor (n = 11). Representative Western blots are shown for CB1, CB2 and GAPDH. Data are normalized to GAPDH are expressed as mean ± S.E.M. (**$p < 0.001$). (C). Immunohistochemical localization of CB1 and CB2 in full term labor and preterm labor amnion.

Fig.2. Effects of THC in human amniotic epithelia cells(WISH) proliferation after 48h of treatment. THC inhibited cell proliferation at 30μM. (** p<0.01 vs. Control) (A). Effects of THC in human amniotic epithelial cells(WISH) migration after 32h of pre-treatment and the cells were allowed to migrate for approximately 16h. THC (10uM) (p<0.05) suppressed cells migration, and THC(20uM) suppressed cells migration completely (* p<0.05 vs. Control) (B, C).

Fig.3. Effects of CB1R inhibitor on THC-inhibited WISH cell migration. (* p<0.05 vs. Control, #p<0.05 vs. THC(10uM) (-),CB1 inhibitor(+))(A,B).

Fig.4. Identification of primary human amniotic epithelial cells(hAECs)(A). Effects of THC in primary human amniotic epithelial cells(hAECs) derived from term labor and preterm labor migration after 32h of pre-treatment and the cells were allowed to migrate for approximately 16h. THC inhibited migration significantly at 10uM and 20μM. (*** p<0.001 vs. Control, ****p<0.0001 vs. Control) (B, C).

Fig.5. Mean relative fold changes of the migration-related genes determined by PCR array following exposure of 20uM THC. PCR array data represent the mean of replicate wells for each treatment group. Fold changes were calculated relative to control samples. Significant fold changes ($p < 0.05$, fold change

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> 2 or fold change < -2) are indicated in this figure(A). The protein level of MMP2 and MMP9 (B,C) in the WISH cells after 48h treatment of THC. (* p<0.05 vs. Control).

Commented [菊蕾2]: 48h treatment with THC

Fig.6. WISH cells were transfected with siRNAs against MMP2, MMP9, or a nonspecific sequence (NC). MMP2 and MMP9 mRNA and protein expression was evaluated by RT–PCR and by Western blot analysis (A, B). Representative immunofluorescence images of siRNA-transfected WISH cells migration (C). Quantification of the number of migrated cells per field transfected with the indicated siRNAs (D).

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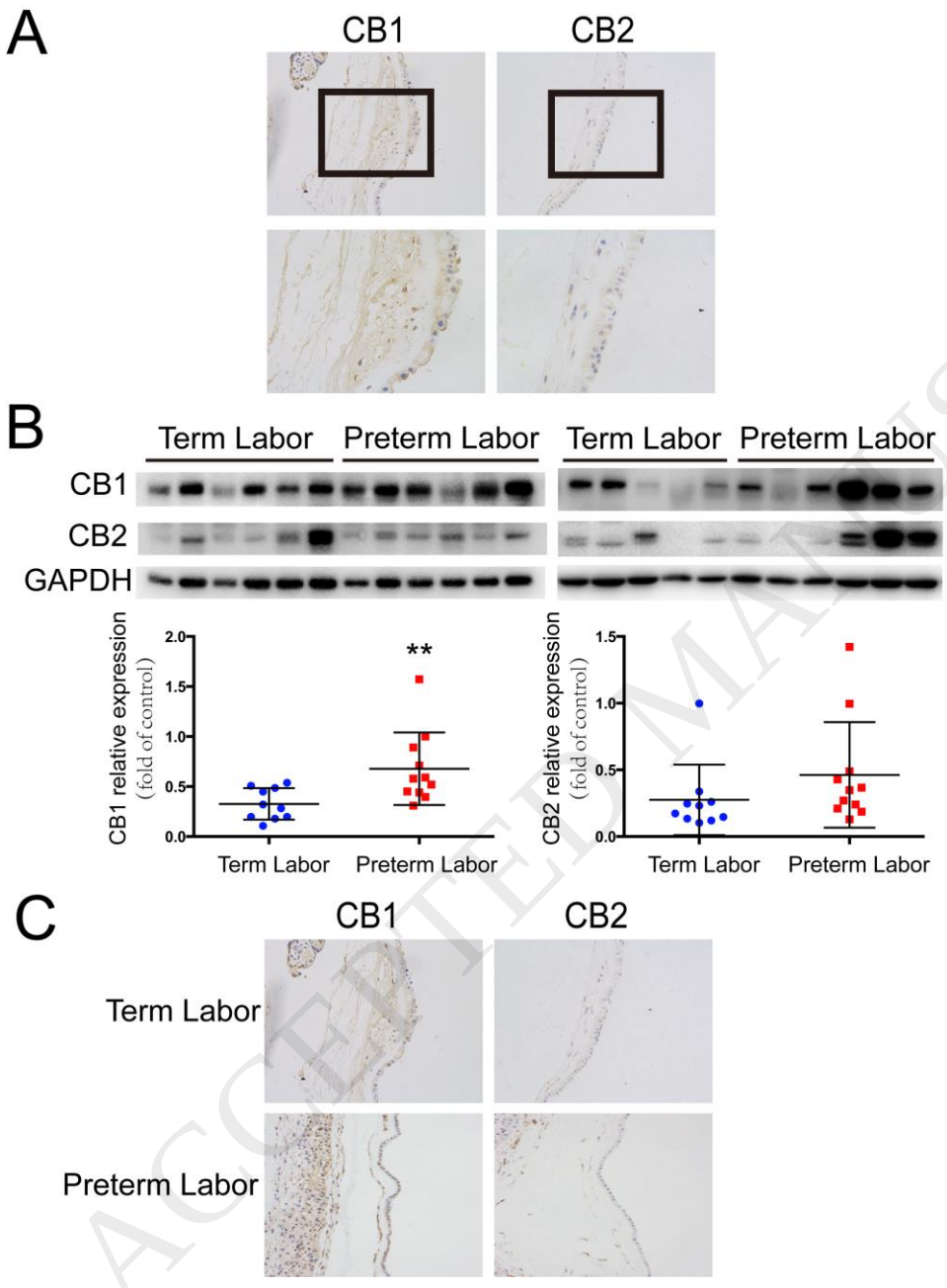

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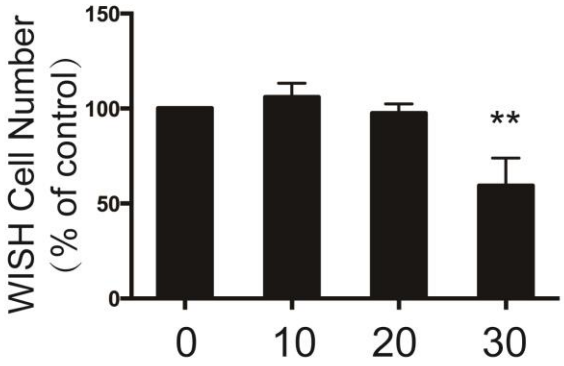

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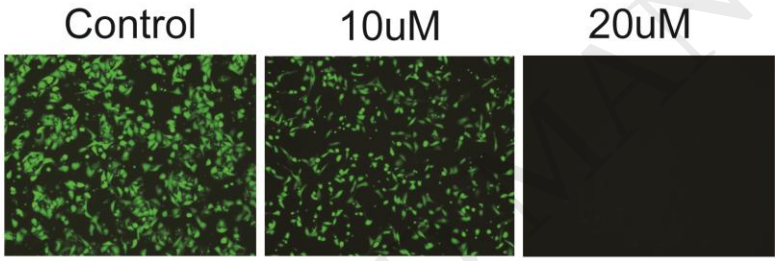

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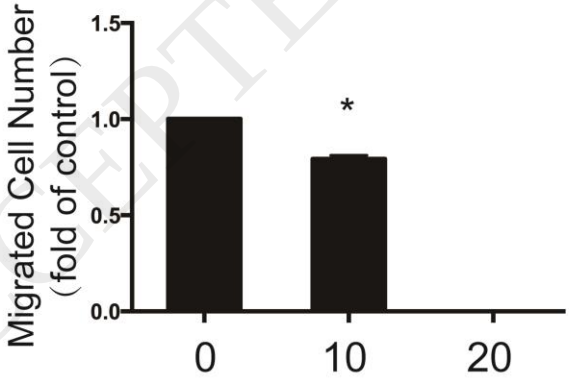

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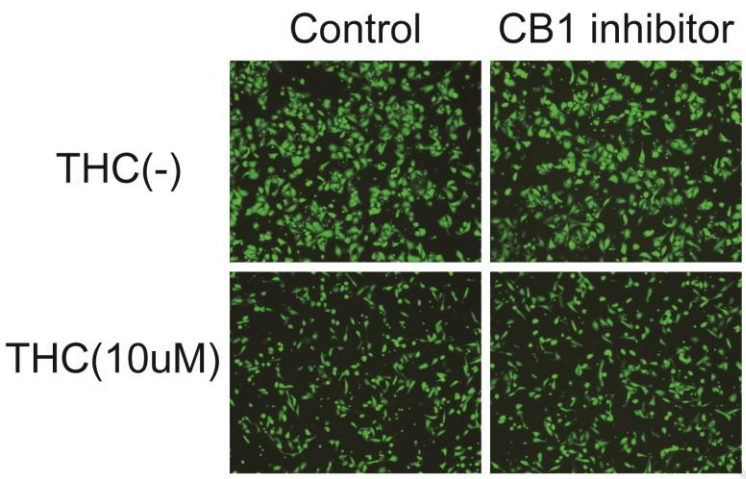

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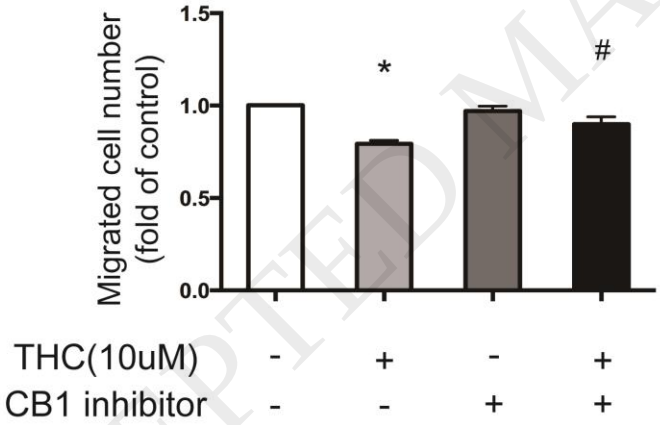

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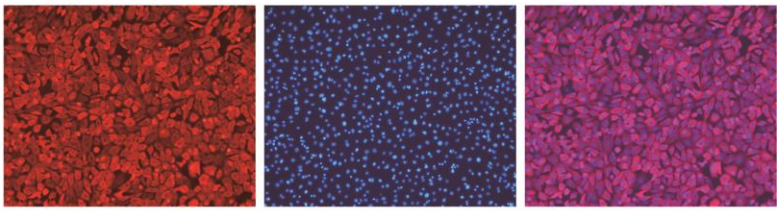

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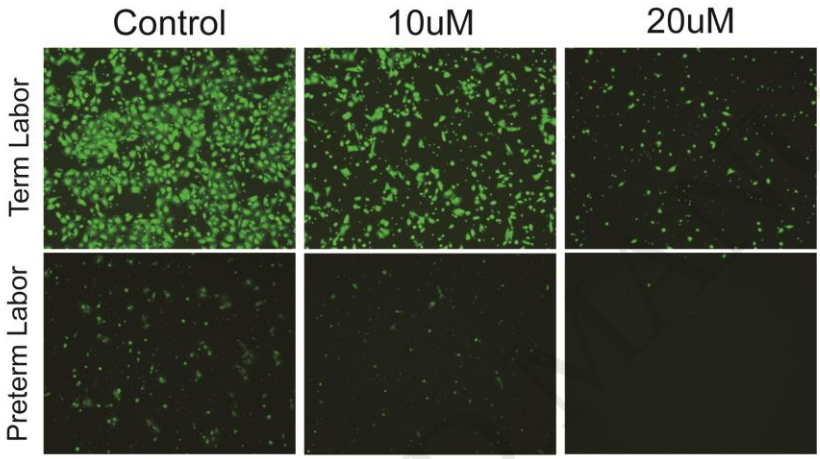

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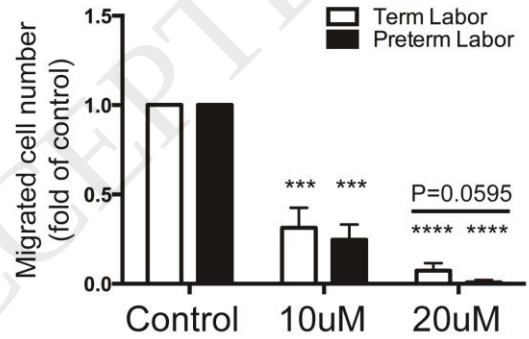

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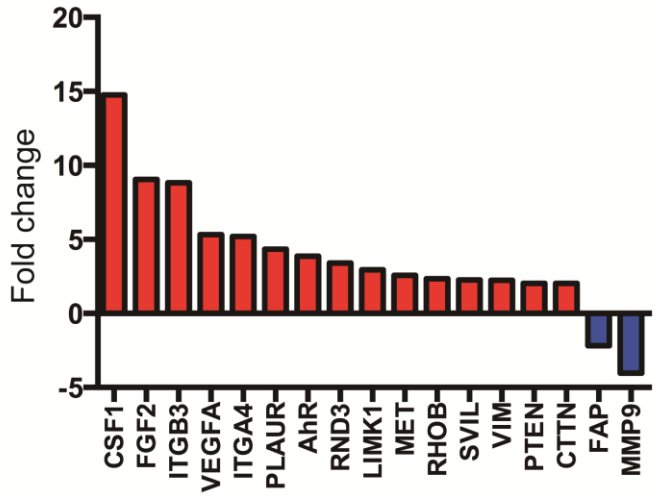

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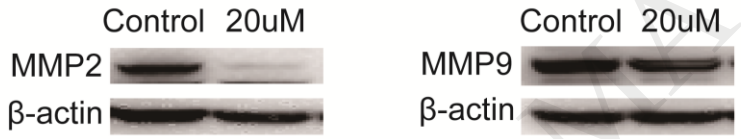

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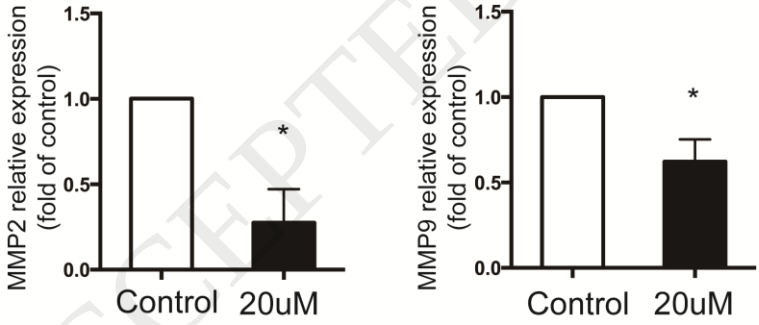

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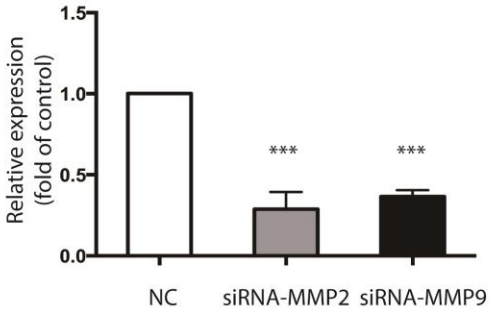

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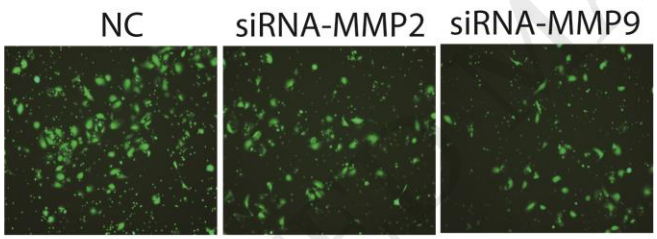

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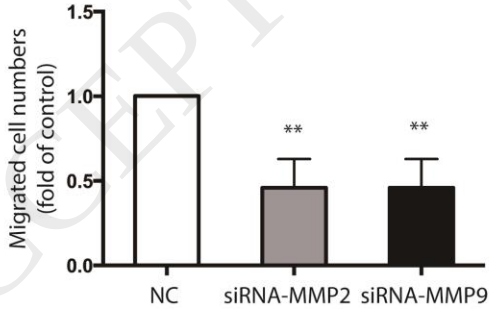

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Table 1. Data expressed as mean (standard error of the mean) or n (%) unless otherwise stated.

P is the P value for the comparison between the preterm groups and the term group.

MOD = mode of delivery.

Table 1

Maternal and neonatal characteristics

	Preterm n=11	Term n=10	P (Preterm v Term)
Gestational age(days)	236.2 ± 7.253	273.3 ± 3.283	<0.001
Maternal age(yrs)	29.00 ± 1.818	27.70 ± 0.9667	0.52
Parity	1.000 ± 0.0	1.100 ± 0.1000	0.36
Gravidity	1.600 ± 0.3055	1.444 ± 0.2422	0.70
Male	6（55%）	4（40%）	0.27
Birth weight(g)	2193 ± 241.7	3337 ± 121.1	<0.001
MOD			
Vaginal delivery	11(100%)	10(100%)	-

Commented [菊蕾3]: Maternal age: the age of the pregnant women when give birth

Preterm: preterm labor, the gestational age of which is less than 37 weeks

Term: term labor, the gestational age of which is between 37 weeks and 42 weeks

MOD = mode of delivery.

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Table 2. The siRNA sequences of the NC, MMP2 and MMP9.

Table 2

	siRNA sequences	
	sense	antisense
NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
MMP2	GCACCCAUUUACACCUACATT	UGUAGGUGUAAAUGGGUGCTT
MMP9	GCGCUGGGCUUAGAUCAUUTT	AAUGAUCUAAGCCCAGCGCTT

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