HIV-1 infection in early human placenta in vitro: Effects of Tumor Necrosis Factor (TNF-α) and Interleukins IL-1 and IL-6

Wu Fei, MD;1# Fan Zhang, MD;2,3# Elizabeth Ding;2,4 Xuejun Kong, MD;2 Jianlin Wu, MD, PhD;1 Ruoyu Wang, MD;1 Zhiqiang Wang, MD;1 Shu Wen, PhD;5 Hong Cao, MD;6 Bin Yang;6 Dewei Zhao, MD;1 Xiang Li, MD;1 Yan Ding, MD, PhD*1,2,7

1 Institute for Translational Medicine, the Affiliated Zhongshan Hospital of Dalian University, Dalian, China
2 Program in Autism Research at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA
3 Shenzhen Hospital Affiliated to Peking University, Shenzhen, China
4 Lexington High School, Lexington, MA
5 Department of Microecology, Dalian Medical University, Dalian, China
6 Genomic Future, Inc., Lexington, MA
7 The Broad Institute of Harvard and MIT, Cambridge, MA

INTRODUCTION
Pediatric AIDS continues to be a national health crisis. Currently an estimated 1.8 million children are living with HIV/AIDS.1 HIV-1 mother-to-infant transmission (perinatal transmission) is the major route of exposure for the fetus/newborn child. It is generally accepted that a range 14% to 40% of HIV positive women in pregnancy will vertically transmit viral infection to their infants in the absence of antiretroviral therapy.1,2 HIV-1 maternal-to-fetal transmission can occur in utero during gestation via transplacental transmission,4 intrapartum near the time of delivery via infected maternal blood contamination,5 or postpartum through breast lactation.6 Among those, between 20 and 50% of perinatal HIV infections occurred in utero during gestation.7,8 HIV-1 provirus DNA has been detected in the fetus tissues as early as 8 weeks gestation9-11. The expression of viral gene products in the villi of placenta from HIV-positive women has been documented.12-14 Several studies using in situ PCR (ISPCR) have demonstrated that placental elements, e.g. trophoblast, Hofbauer cells can be infected by HIV-1.15-20 However, there is conflicting evidence regarding the susceptibility of the trophoblast cells to HIV infection. Some studies demonstrated that HIV gene products was in trophoblast as well as in placental macrophages (Hofbauer) of the villous stroma, others found only in Hofbauer cells.21-23 Controversy extends to in vitro investigations by using primary trophoblast preparations and trophoblast cell lines, which have been transiently infected by free virus.24-27 In contrast, other investigators have been unable to demonstrate any trophoblast infection with cell-free virus, even at a high multiplicity of infection (MOI).28-30 Thus, whether the trophoblast is a barrier that prevents entry of free virus or

The placenta plays a pivotal role in the pathogenesis for the vertical transmission of HIV-1 during pregnancy. To understand HIV-1 infection during the first trimester of pregnancy and its association with inflammatory cytokines, we developed an in vitro HIV infection model using the early human placental explant cultures (5.5-7.5 wks). Ba-L, a laboratory-adapted, chemokine receptor CCR5-using strain of HIV-1, was used to infect the explants. DNA-PCR and Southern blot results show that HIV-1 is able to infect the early human placenta in a viral load-dependent manner. In situ PCR demonstrated that the majority of HIV-1-infected cells are cytotrophoblast, Hofbauer cells, and with few syncytiotrophoblast. Viral protein p24 and reverse transcriptase (RT) increase by approximately three-fold in the culture supernatants when exogenous cytokines tumor necrosis factor-alpha (TNF-α) alone or in combination with interleukin-1-beta (IL-1β) and IL-6 were added to these virus-exposed explants. Taken together, these data support the hypothesis that the early human placenta is susceptible to HIV-1 and latent vs. active HIV-1 infection in human placenta may be an important factor in determining whether the baby becomes infected.


Key Words: HIV-1, virus infection, trophoblast, in situ PCR, human placenta
actively promotes its dissemination into fetal tissues is not clear. Defining the exact role of the placental trophoblast in vertical transmission of HIV has become imperative since HIV DNA has been demonstrated in more than 70% of placenta from HIV-infected women.\textsuperscript{31}

Latent viral infection after acute viral exposure is the common mode of most virus infection. HIV-infected cells can harbor the virus in a state of relative or absolute latency.\textsuperscript{32} Previous studies have shown that several inflammatory cytokines play an important role in the host-mediated control of HIV-1 replication.\textsuperscript{32} Inflammatory cytokines, TNF-\(\alpha\) or IL-1 plus IL-6, for instance, induce HIV expression via activation of the cellular transcription factor NF-kB\textsuperscript{33,34} or posttranscriptional modifications. The association between placental inflammation and increased transmission of HIV-1 during pregnancy\textsuperscript{35,36} suggests that inflammatory cytokines enhance infection of fetal tissues. Characterization of HIV infection in the early human placenta will be helpful for designing novel strategies to label event early placental infection and subsequent transplacental transmission to the fetus. In the present study, we used an in vitro 6-8 weeks first trimester human placenta culture to identify HIV-1 in vitro infection in the early human placenta and its response to the exogenous stimulatory cytokines.

**MATERIALS AND METHODS**

**Tissue collection and processing.** Five first trimester human placenta (5.5-7.5 weeks of gestation) were collected from pregnancies terminated of healthy HIV-1-negative patients for psychosocial reasons from the Affiliated Zhongshan Hospital. A signed consent was obtained from each patient and the institutional review board of Affiliated Zhongshan Hospital approved the study. Tissue was collected directly in cold, sterile phosphate-buffered saline (PBS) and transported to the laboratory on ice. Placenta were rinsed with PBS and dissected under a stereomicroscope into characteristic tree-like villous explants. Lymph nodes were from patients who died from AIDS.

**In vitro HIV infection with placental explants.** *in vitro* placental explant 3-D culture model that allows the outgrowth of proliferative extravillous trophoblasts was developed as described by Genbacev et al.\textsuperscript{37} Briefly, explants of \(\sim\)10 mg wet weight were individually placed in 12mm diameter Millicell-CM culture dish inserts (Millipore Corp., Bedford, MA) layered with 200\(\mu\)l Matrigel (Collaborative Research, Bedford, MA). Inserts were placed in 24 well culture dishes (Costar, Cambridge, MA). Nutrient media F12 HAM (Sigma) supplemented with antibiotic/antimycotic powder (Sigma) was used in all experiments. Media containing 20% fetal bovine serum (FBS) (Sigma) was used in a volume of 1 ml per culture well (0.7ml bottom well, 0.3 ml upper well) and was changed daily. Cultures were inspected daily under an inverted phase contrast microscope (Nikon ELWD 0.3) for the appearance of proliferative outgrowth. Additional viability index of the tissue has also been established using glucose consumption, lactate and placental hormone production. When the outgrowth of EVT is visibly detected after about 36 hours culture, the supernatant medium was replaced by medium containing HIV-1/Ba-L at concentrations of 250-, 500-, 1000- and 2000-fold of the minimal infection dose defined in PBMCs. The exposure to HIV-1 virus continues for 24 hours, and then aspirated and rinsed with PBS (pH 7.4) five times until residual virus was completely washed out. Then 1 ml fresh medium was added to each well, and continued culture for up to 6 days at 37°C, 5% CO\(_2\). To determine if stimulatory cytokines affect virus production in HIV-1-infected placental explants, either TNF-\(\alpha\) alone or the combination of IL-1 and IL-6 was added in the culture medium along with virus or after 24 hour virus exposure. The culture medium was changed every 24 hour and the supernatant was stored at -80°C freezer for bioassays. One part of explants was removed and frozen in -80°C freezer for PCR analysis and Southern Blot, the other part of explant was fixed in 10% neutral buffered formalin (Sigma). Then the tissues were immersed in 70% ETOH stored at 4°C until processed through a series of ethanol and xylene, and embedded in paraffin.

**DNA-PCR and Southern Blot analysis.** DNA was extracted from explant samples using the QIAamp DNA Mini Kit from QIAGEN. DNA concentration was determined by Pharmacia Biotech GeneQuant RNA/DNA calculator and all samples were diluted to 100 ng/\(\mu\)l. Hot start PCR was used to detect the GAG region of HIV DNA. The primers used were as follows: SK145 (5'-AGTGGGGGGCATAAGGGAAGACATCAAGCAGCCA-TGCAAAT-3') and SK431 (5'-TGCTATGTCAGTTCCCC-TTGGTTCTCT-3'). PCR was carried out in a 50 \(\mu\)l reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCL, 2.5 mM MgCl\(_2\), 0.2 mM each dNTP, 0.5 each primer (Sigma-Genosys), 1.25 U of Taq DNA polymerase (Gibco BRL) and 2 nM TaqStart antibody (Clontech). Cycling conditions for the GAG reaction were initial denaturing 5 mm at 95°C then 40 cycles of 15 sec at 94°C, 15 sec at 62°C, and 30 sec at 72°C, then a final extension of 7 min at 72°C. All thermocycling was done on a Thermolyne Ampliptron II. 500 ng of DNA was used in each and 10 \(\mu\)l of amplified product was electrophoresed on a 2% agarose gel with ethidium bromide and visualized under UV light. Controls were 8ESLav cells of known quantity diluted in PBMCs and then DNA was extracted and used for PCR. The amplified fragment length is 142 bp. Specificity of the HIV gag sequence was verified by pressure blotting of the PCR products onto a nylon membrane (Roche), followed by overnight hybridization with a [\(\alpha\)-\textsuperscript{32}P]dATP-tailed oligonucleotide probe SK102 (5'-GAGACCATCAATGAG-GAAGCTGCAGAATGGGAT-3'). Then a wash with a 0.1xSSC buffer and 0.1% (w/v) SDS wash solution at 60 °C for 30 minutes was carried out for a high-stringency wash. Product was detected by autoradiography using Biomax-MS film with an intensifying screen.

**In situ PCR.** *In situ* PCR was performed using direct biotin-16-dUTP incorporation into HIV-1 DNA-PCR products as described by Strappe PM, et al.\textsuperscript{13} with minor modifications. Briefly, sections were digested by 6 \(\mu\)g/ml proteinase K in 0.1 M Tris-HCL, 5 mM EDTA, pH 7.5 for 20 min at 37°C.
Tissue sections were microwaved without boiling for 15 min in citrate buffer. Slides were then quenched in 3% Hydrogen peroxide in methanol for 10 min at 4°C. PCR was done with the SK145 and SK431 primers using the following cycling parameters: 95°C for 3 min then 21 cycles of 94°C for 30 sec and 59°C for 1 min on a PTC 100 M.J. Research thermocycler. The solution for PCR amplification is contained with 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 2.5mM MgCl2, 0.2mM each dNTP, 20µM of Biotin-16-dUTP, 0.5 µM each primer (Sigma Genosys), 0.1U/µIU of Taq DNA polymerase (Roche), and 2nM TaqStart antibody (Clontech). A post-PCR wash was done at 40°C for 5 min in 2X SSC. The sections were fixed in 100% ethanol for 15 min. The PCR product was detected by treating the sections with Vector Elite ABC-HRP followed by DAB substrate (Vector). The sections were counterstained with Methyl green and coverslipped. Controls for in situ PCR included reactions without primers, a reaction containing no HIV-1 gag, a reaction without Taq-polymerase, and a reaction without biotin16-dUTP. Appropriate controls for HIV-1 in situ amplification with HIV-1 positive and negative lymph nodes, and an internal positive control for in situ amplification with β-globin primers 5’-ACACAACGTGTCCATC-3’ (14-33) and 5’-CAACTTCATCCA-GTGTACC-3’ (104-123) were also carried out. To ensure that the positive in situ PCR signal was not due to diffusion of amplified DNA to an uninfected cell, the post in situ PCR amplification solution was electrophoresed on a 2% agarose gel with molecular weight markers and stained with ethidium bromide.

p24 core protein assay. p24 core antigen of HIV-1 was assayed using the Vironostika (Organon) enzyme immunoassay based on the “sandwich” principle. Anti-HIV-1 (human) coupled to horseradish peroxidase (HRP) serves as the conjugate with tetramethylbenzidine (TMB) as substrate. Assay was performed according to the manufacturer instructions. The absorbance of p24 was read by automatic microplate reader and analyzed by SofMax™ Software program. p24 was expressed as pg/mg of tissue/24h. Samples from HIV uninfected placental cultures were used as negative control.

Figure 1. Viral concentration and its association with placental explants infectivity. Percentage of HIV-1 positive explants by DNA-PCR was calculated on day 6 of culture. Bars indicate the means of 12 explants from each of 5 different placenta. Y-axis: values were calculated as a percent of positive explants over total explants exposed to virus. All are significantly different from 250-fold. * Significantly different from 250-, 1000-, and 2000-fold of minimal infectious dose defined in PBMCs (p<0.05). (N=12, number of placenta = 5)
Reverse transcriptase (RT) activity assay. The RT activity was carried out using RT assay kit (Retrosys, Invitrogen) according to the manufacturer's instruction. Briefly, 50 ml of serially diluted sample (1/5, 1/25, and 1/125) is added to a 96-well microtiter plate, with poly(rA) coupled to the bottom of the wells as the enzyme template and 150ml of reaction solution consisting of oligo(dT) as the primer and BrdUTP as the deoxynucleoside triphosphate substrate. Sample dilution buffer for direct RT activity determination consists of HEPES (10 mM), MgCl2 z 6H2O (4 mM), dextran sulfate (50 mg/ml), and Triton X-100 (1%). RT, if present in the sample, catalyzes the polymerization of a new DNA strand along the poly(rA) template consisting of incorporated bromodeoxyuridine monophosphate. Polymerization is allowed to proceed for a desired period of time, after which the plate is washed to remove unused substrate. In our case we used an overnight(15h) polymerization step. Product detection is carried out immunologically by adding alkaline phosphatase (AP)-conjugated anti-BrdU Ab to each well for 90 min at 33°C. The plate is then washed, and this is followed by a colorimetric determination of the bound Ab with an AP substrate, para-nitrophenyl phosphate. The intensity of the color reaction is read in a standard plate reader (405 nm) using a microtiter plate reader. The amount of RT present in each sample is expressed as pg/mg of tissue/24h) by using a serially diluted reference enzyme with a known RT concentration on all plates. The concentration in each sample dilution is then used to calculate the mean concentration in the undiluted sample. Samples from uninfected placental cultures were used as negative control.

Statistics analysis. Each condition was in triplicate, and results were analyzed for statistical significance by analysis of variance (ANOVA) using the software StatView 512+ (Brain Powers, Inc). Post-hoc comparisons were made using Scheffe’s F-test alpha set at 0.05.

RESULTS

Viral concentration and placental explant infectivity. To determine whether placental cells are permissive for HIV-1 infection and the correlation between viral concentrations and explant infectivity. Culture supernatants were replaced by medium containing HIV-1 virus (Ba-L) at 250-, 500-, 1000-, and 2000-fold of minimal infectious dose defined by PBMCs in Ham-F12culture media for24 hours. Then, explant cultures were intensively washed with fresh culture medium. Explants

Figure 2. Southern blot analysis of HIV-1 DNA extracted from HIV-infected placental explants. HIV-1-positive standard 8E5 Lav, distilled water and uninfected placental tissue were used as controls. A 500 ng amount of each denatured DNA sample was used for PCR, using primers SK145 and SK431 from the gag region of HIV-1 DNA. PCR products were denatured and subjected to hybridization with a specific 32P-end-labeled (SK102) probe. Lanes 1 to 3, HIV-1 positive standard 8E5 Lav 100, 10, and 1 copies; lanes 4 and 5, PCR negative control with dH2O; lanes 6 and 7, PCR of DNA isolated from HIV -unchallenged placental tissues; lanes 8 and 9, PCR of DNA isolated from HIV-positive placental tissues.
were continued to culture for up to 6 days. Cultures without virus exposure serve as negative controls. Explants were taken out for DNA extraction followed by DNA-PCR for HIV-1 gag region. The primers were as follows: SK145 (5’-AGTGGGGGCACTAACGAG-CCATGCAAAAT-3’) and SK43 (5’-TGCTATGTCAGTCCCTTGGTTCTCT-3’). Preliminary results show that with the increase of viral concentration from 250- to 2000-fold of minimal infectious dose defined in PBMCs, the infectivity rates increased from 10% to 100% (explants numbers =12, 6 cultures) (Figure 1). Thus, viral dose at 1000-fold almost consistently infects the explants. This result shows that HIV is able to infect the early human placenta \textit{in vitro}, and there is a positive correlation between viral load and placenta infectivity in vitro, which is consistent with the clinical observation on the correlation between viral load and rate of vertical transmission.\textsuperscript{38}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{\textbf{Figure 3.} ISPCR of HIV-1 DNA in the early human placenta. \textit{In situ} PCR for HIV in the early placental explants cultured on matrigel for six days. DAB stain (brownish color) is localized in cells infected with HIV. a and c: placenta infected with HIV-1 \textit{in vitro} that HIV-1 positive staining of trophoblast cells were primarily observed (arrowheads) in: CT = cytotrophoblast, EVT extravillous trophoblast, and in some Hoflauer and dendritic cells, but not in ST = syncytiotrophoblast. b: HIV-1 unchallenged placenta. d: placenta treated with the omission of SK145 and SK431 primers as the negative controls. e: Lymph nodes from the patients with HIV-1 infection as positive control. Note that the nuclear positive staining (brownish color) in most of the T-lymphocytes. f: Lymph nodes from HIV-1 negative patients as negative control. Section is counterstained with Methyl green. (Magnification: 40x, number of placenta =5)}
\end{figure}
Figure 4. Effects of cytokines TNF-α and IL-1 plus IL-6 on HIV-1 infection in the early human placenta. HIV-1 virus production was evaluated by measuring p24 protein and RT activity in the culture supernatants from the first trimester placental explants. The tissue cultures were exposed to the viral inoculums (Ba-L, 1000x) in the absence or in the presence of IL-1 plus IL-6 (1 ng/ml) and TNF-α (100 units/ml) for 24 hours. Then the cultures were intensively rinsed with fresh medium HAMF 12 to completely remove the excessive viral residual. 100 µl of culture supernatant was removed from each culture every 24 hours to assay for HIV-1 p24 antigen and RT activity by micro-plate ELISA. Results are expressed in pg/mg/24h of wet placental tissue (mean ±SE). *Values of IL-1β plus IL-6 and TNF-α treatments are significantly different with comparison of virus only and control (P < 0.05); there is no significant difference between of IL-1 plus IL-6 and TNF-α treatments. (Number of placenta = 5)
Detection of HIV-1 DNA sequences in the infected early human placenta. To demonstrate that the HIV-1 RNA genome was actively transcribed into DNA in infected placental tissues and its integration specificity, we analyzed DNA extracted from HIV-1-infected and uninfected samples by DNA-PCR followed by Southern blot analysis on their PCR products. Figure 2 demonstrates the presence of the amplified 142-bp gag region of HIV-1 DNA in infected placenta.

**in situ** PCR detection of HIV-1 DNA localization in the early human placenta. To further identify which cells within the placenta were HIV-1 infected, *in situ* PCR was used to detect HIV-1 proviral DNA in formalin fixed, paraffin embedded HIV-1-positive explant tissues. A combination of microwave irradiation of tissue sections and the use of a two-step PCR ensured that no primer independent false positive signals were detected. ISPCR for HIV-1 DNA in the early placenta shows that HIV-1 DNA positive nuclei primarily localized in EVT (Figure 3a), cytotrophoblast (CT), and Hofbauer cells, but few in syncytiotrophoblast (Figure 3c). While Figure 3d shows that the result of ISPCR for HIV-1 DNA in HIV unchallenged placenta was completely negative. Negative controls included amplification reactions with the omission of SK145 and SK43 I primers (Figure 3d), Taq-polymerase and biotin-labeled dUTP. ISPCR for HIV-1 DNA was also performed on known HIV-1 positive and negative lymph nodes (Figure 3e and Figure 3f), results show that the majority of HIV-1 positive cells in lymph node are T-lymphocytes or macrophages based on the morphological appearance and IHC for CD3 and CD68 receptors (data not shown). The negative control demonstrates that the primers SK145 and SK43I are not binding to non-specific targets, that there is no polymerase DNA repair reaction and that the detection systems are not creating false positives due to background staining. Possible chance of the positive localization due to cell-cell diffusion of PCR product was also ruled out by agarose gel electrophoresis of the post in situ PCR solution, since the post-PCR solution was negative for HIV-1 DNA (data not shown).

HIV-1 virus production and its response to cytokines Figures 2 and 3 have clearly shown the HIV-1 provirus expression as well as cellular localization in placental tissues. However, what is the infection status of HIV-1 in placenta, in active virus production or a latent stage of infection? To address this critical issue, time-course studies with two major indexes for active HIV-1 virus production, core protein p24 release and reverse transcriptase activity, in the placental explant culture and its response to stimulatory cytokines were carried out. As shown in Figure 4a, p24 protein production after 24h virus exposure was at a level of 40 pg/mg/24h. However, after the second day of post-infection, the p24 protein production after 24h virus exposure was at a level of 40 pg/mg/24h. However, after the second day of post-infection, the p24 production was decreased down to control level, and remained at the same level up to 6 days post-infection culture. Furthermore, the kinetics of reverse transcriptase activity in the infected placental tissues showed a similar pattern to that of p24 productions (Figure 4b). Additionally, when co-cultured with phytohemagglutinin-stimulated peripheral blood mononuclear cells up to 28 days, supernatants obtained from HIV-infected explant cultures did not produce HIV-1 (data not shown). Taken together, data indicate that the HIV-1 infection with placenta explant was at, if not a latent stage, an extremely low level. To further investigate if stimulatory cytokines can activate the latent HIV-1 virus replication in placental tissues, either TNF-α alone or the combination of IL-1 and IL-6 was continuously included starting the same time the virus was added. As noted in Figure 6, these stimulatory cytokines dramatically increase viral release after the second day of post-infection as reflected in p24 and in RT compared with control (p < 0.05), and enhanced the virus production approximately 3-fold after the 6-day of post-infection culture.

**DISCUSSION**

*In vitro* HIV-1 infection and its response to cytokines in the early human placenta were studied. An *in vitro* placental explant HIV-1 infection model was developed using matrigel culture system. This culture system is unique since it allows the proliferation of extravillous trophoblasts, which arise from the out-breaking cytotrophoblast cells from the tips of villous. To determine if the early human placenta is susceptible to HIV-1 and the association of HIV-1 infection with viral concentration, DNA-PCR of HIV-1 provirus followed by Southern Blot on the PCR product were carried out. As shown in Figures 1 and 2, the early human placenta was indeed infected by HIV-1, and its infectivity is positively correlated to the viral concentration. Results here provide direct evidence for HIV-1 infection in the early human pregnancy. However, we want to know not only human placenta can be infected, but also what cells in the placenta were in fact infected. Understanding the localization of HIV infected cells in placenta is essential for the understanding the pathogenesis of HIV-1 vertical transmission. Previous studies have demonstrated that full-term human placenta can be infected by HIV-1 both *in vivo* and *in vitro* using *in situ* PCR. ISPCR overcomes the problem of inadequate amounts of the target for *in situ* hybridization and retains the advantages of *in situ* hybridization for localization of a specific target at the cellular level. An ideal approach to ISPCR would be using an organ culture system that closely resembles the *in vivo* tissue organization, controlling exposure to virus and then comparing detection of infection with standard technique.

ISPCR of our present study on the first trimester human placenta shows (Figure 5) that trophoblasts cells, especially EVT and CT are strongly positive, but ST was only weakly positive, suggesting that EVT and CT are the primary trophoblasts to be infected by HIV-1. In addition, Hofbauer cells are also stained positive, which is consistent with previous findings that Hofaburer cells are susceptible to HIV-1 infection. This result is different from previous findings in that ST in full-term human placenta was the major cell type for HIV-1 DNA positive staining using ISPCR. One
might question how infection occurs without ST infection. It looks odds initially, since, ST, from the anatomic view of point, is localized on the boundary between maternal blood circulation and fetal mesenchyma. If that EVT and CT in the early human placenta are the major targets of HIV-1 infection is true, argument may arise as to how HIV-1 gets into the CT and stroma while bypass the ST layer. There are several potential explanations for this argument.

First, EVT is derived from the out-breaking of CT from the villous tip. At the early stage of gestation EVT can invade into the maternal endometrium as a placental anchor, or float in the mother blood circulation. HIV-1 may be transmitted through the cytotrophoblast from the infected EVT although this needs further investigation. In addition, previous studies have shown that productive HIV infection depends on the proliferation or differentiation stage of the target cells. However, term trophoblasts do not proliferate in vitro, HIV infection was not dependent on the differentiation of CT into ST, and no study has ever been done for the trophoblast cell proliferation in HIV infection. Results of our present study support the idea that proliferation of trophoblast cells may play an important role in HIV-1 infection. It may also partially explain the recent demonstration of HIV provirus in >70% of placenta from HIV-infected women which suggests the ubiquitous HIV-infection of placenta from HIV-1-infected women.

Secondly, several previous studies as well as our recent studies on CD4 distribution in the early human placenta have consistently shown that the majority of CD4 positive cells in placenta are CT and occasionally in Hofbauer cells. Recently, the authors have evaluated the chemokine receptor localization in the first trimester human placenta. Certain chemokine receptors act as co-receptors for HIV-1 cell entry and are thought to be essential for HIV infection. Our data show CCR5 in EVT and cytotrophoblast cells, but not in ST. This data adds additional evidence that EVT, but not ST, may be essential for HIV placental infection. Thirdly, HIV may cross the ST layer by transcytosis. It has been demonstrated that HIV-1 crosses the endothelium into intestine by this mechanism. Finally, differences in HIV-1 infection (full-term versus 6-8 weeks first trimester) may be due to the different gestation stage of placental tissues used or different in vitro culture conditions (matrigel culture versus suspension culture) were applied.

Several investigators have examined possible roles of placenta, and trophoblasts in particular, in HIV-1 mother-to-infant transmission. Unfortunately there is a lacking of consensus in regard to what role, if any, is played by trophoblast cell in HIV vertical transmission. In the literature, both preventive and facilitated effects have been ascribed to trophoblast cells. Thus, whether the trophoblast is a barrier that prevents entry of free virus or actively promotes its dissemination into fetal tissues is not clear, and efforts to reconcile these differences have been minimal. Several studies have demonstrated that human placental trophoblast cell lines or choriocarcinoma-derived trophoblast as well as Hofbauer cells can be infected with HIV-1. In contrast, conflicting evidence regarding the susceptibility of trophoblast to HIV infection were also reported by some authors suggesting that no trophoblast can be infected with cell-free virus, even at a high multiplicity of infection. However, a careful examination of the in vitro human trophoblast cell culture they used reveals that their cell isolation is achieved by extremely harsh treatment with digestive enzyme (DNase, trypsin, or collagenase). Consequently, it is difficult to rule out that some cell properties was not modified during the process of isolation. Thus, the isolated primary trophoblast culture does not become easily infected and are not a good model for studying HIV infection as noted in their references. Nevertheless, it would appear that the role of trophoblast is best studied in tissue organ systems like placental explants, which as in our case, could maintain the normal cell-cell and cell-matrix interactions and are more likely to reflect the state.

Risk factors associated with HIV mother-to-infant transmission including women with low CD4 cell counts, high viral load, chorioamnionitis, vitamin A deficiency, smoking and co-infection with hepatitis C have been reported, although remain controversial. Probably, the higher viral load associated with immune depletion and with early infection is a major determinant of the degree of infectiousness. However, only about one-third of the reduction in vertical transmission can be explained by reduction in maternal HIV plasma viral load. There is no viral load threshold below which perinatal transmission has not occurred. Similarly, there is no threshold above which transmission always occur.

Under normal condition of pregnancy. HIV-1 infected placenta may remain in a latent stage of infection. This low replicative potential may be the result of the protective effect of interferons (IFNs) produced by trophoblast cells. Human placental trophoblast cells normally possess high levels of IFN-induced 2,5'-oligoadénylate (2-5A) synthetase activity, which can be augmented by HIV 5 RNA. Moreover, the specific cellular localization of this enzyme suggested that the 2-5A synthetase system negatively regulated HIV replication in the trophoblast cell. In particular, IFN-alpha has been demonstrated in syncytiotrophoblast and cyt trophoblast cells and is credited with preventing cell-mediated and cell-free HIV infection of trophoblast cells in dose-dependent manner. Viral replication may be influenced by a variety of cytokines. It has been demonstrated that some cytokines, like TNF-α, IL-1 and IL-6, secreted from placental macrophages and trophoblast cells can up-regulate HIV-1 expression in T cells and in a promonocyotic cell line. This up-regulation of HIV-1 expression may provide a mechanism through which latently infected fetal and maternal lymphocytes and placental macrophages become activated and subsequently release productive virus. Both in vivo and in vitro studies have demonstrated that TNF-α plays a critical role in HIV pathogenesis. It has been reported that TNF-α can drive virus replication in activated lymphocytes and macrophages and
PBMCs, and it appears to up-regulate HIV-1 expression by activating the important cellular transcription factor, NF-kB. Investigators have also demonstrated that IL-6 can induce HIV replication in chronically infected monocyte cultures and can synergize with TNF-α. The mechanism of IL-6-mediated HIV induction is not fully understood, but its effect is most likely posttranscriptional. When IL-6 and TNF-α interact synergistically to induce HIV expression, both transcriptional and posttranscriptional pathways are involved. IL-1β directly induces expression of HIV in monocyte cell lines and synergizes with IL-6 to upregulate virus expression. IL-1 is thought to induce HIV expression at the viral transcription level in a NF-κB-independent manner, predominantly by posttranscriptional mechanism. It is tempting to speculate that the presence of these cytokines in the placental microenvironment plays a regulatory role in either the expression of HIV or the protection of the host although the mechanism(s) by which latent virus in the placenta develops into a fulminating infection remain to be defined. Our studies show that either TNF-α alone or the combination of IL-1 and IL-6 dramatically enhance the latent virus replication in HIV-1 infected placenta (Figure 6a and 6b). Thus, one can argue that the production and transcription of IL-1 beta, IL-6, and TNF-α have potential of creating a placental microenvironment that is conducive to the activation of a latent infection or the expansion of a low-grade infection that may facilitate vertical transmission of the virus. Our result lends strong support to this possibility. Studies from other group using full-term placenta tissues for HIV-1 infection have also shown positive HIV-1 DNA in trophoblast cells, however, the effect of cytokines on the infection was not investigated.

The influence of placental resource difference may be ruled out since more than five placental explant cultures obtained from different women were used in this study, and results were very well reproducible. If in utero infection is dependent on placental infection, recognition of an affected placenta could provide a mechanism for identification of at-risk infants subsequently identified as HIV-1 infected. Identification of both viral factors like different co-receptor - using virus strains and placental factors like chemokine receptors expression relating to HIV-1 infection of placenta and subsequent transplacental transmission needs further investigation. We anticipate that this early human placenta matrigel culture would become an ideal model for future in vitro anti-HIV therapy study, especially in screening safety drugs for HIV-1 mother-to-fetal transmission intervention.

ACKNOWLEDGEMENTS
We thank Dr. Tim Lilburn for his helpful discussion of the results and careful proof reading of the manuscript. Innovative - The research was partially funded by the Grant from Chinese Central Government of Human Resources (YD), and Merit Grant for Extraordinary Oversea Talent from Dalian Government, China (YD).

CONFLICT OF INTEREST
None.

REFERENCES


44. Ding Y. Distribution of the human immunodeficiency virus receptor CD4 and chemokine receptors: CCR5, CXCR4, and CCR3 in the early human placenta. J Immunol. in press.


59. Duh EJ, Maury WF, Folks TM. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. Proc Natl Acad Sci USA. 1987;84:5974-5979.


