行政院國家科學委員會補助專題研究計畫成果報告

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侵襲性滋養層細胞於著床後對子宮螺旋動脈血管重塑扮演角色之探討

The role of invasive extravillous trophoblast in the remodeling of spiral artery during normal and abnormal implantation

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計畫主持人：施景中

共同主持人：謝豐舟　錢宗良

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行政院國家科學委員會專題研究計畫成果報告

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一、中文摘要

成功的人類著床倚賴絨毛外滋養層細胞透過增殖、轉移、侵入母體的蛻膜，並適度的轉換血管內皮細胞，以達成有效建立子宮胎盤循環。可是至今，這些滋養層細胞如何引發並執行母體子宮螺旋動脈轉換及血管重塑，真正機轉仍不明朗。一個重要的因子為這些滋養層細胞侵入血管管腔內，並跨站於原來的內皮細胞上將其取代轉換，目前的主流觀念為 Hamilton 於 1965 年於 Nature 中所提出，這些侵襲性滋養層細胞來自基本板，由子宮螺旋動脈的末端逆流而上，並非由蛻膜的間質區裡的侵襲性滋養層細胞所侵入。這個主流觀念衍生一些重要問題尚未能解答，第一，若是如此，蛻膜的間質區裡的侵襲性滋養層細胞於人類著床中所扮演的角色為何，為什麼這些滋養層細胞要千里迢迢至母體胎兒的交界面—細胞板，穿越母體的蛻膜，最後有些遠遠地落腳在子宮肌肉層裡？第二、何者觸發基本板的滋養層細胞去對抗動脈高壓、逆流而上？

蛻膜的間質區裡的侵襲性滋養層細胞，早先被認為與人類著床的免疫調節有關，其實它的真正角色從未被清楚確定。晚近，有學者發現到這些細胞自脫離了細胞板後，大量集結在蛻膜血管的周遭，這些學者因此假定：這些細胞極有可能在子宮螺旋動脈的血管重塑扮演重要角色，可能是集結在蛻膜血管的周遭後，與血管產生互動，進而放出訊號使基本板的滋養層細胞去對抗動脈高壓、逆流而上侵入血管。事實上侵襲性滋養層細胞侵入母體組織也血管內滋養層細胞空間較深遠而且時序上也較早，這些重要的著床發育上的問題正是本計劃欲探討的主軸。

在本計劃的第一年裡，我們收取早期妊娠流產手術的蛻膜組織，以及足月正常妊娠及子癲前症的胎盤底部採樣(剖腹產時取)，我們進一步將以免疫螢光反應、共軛焦距螢光顯微、掃瞄式電子顯微鏡、穿透式電子顯微鏡、免疫電子顯微鏡，去觀察原位標本下，侵襲性滋養層細胞(含侵襲性滋養層細胞、血管內滋養層細胞、多核滋養層巨細胞)本身、及與著床微環境互動下的超微結構變化(因細胞執行既定命令時仍須透超微結構改變) ，這些觀察將對滋養層細胞著床提供重要資訊。本年計劃的亦將體外培養及分離滋養層細胞以供第二年實驗之用。

關鍵詞：侵襲性滋養層細胞，著床，血管重塑，人類，細胞生長因子。

Abstract

Successful human placentation depends on adequate transformation of the uteroplacental circulation by extravillous trophoblast proliferation, migration, and invasion into the maternal decidua. Thus far, how these trophoblasts execute the process of spiral arteries transformation is still not clear. One key factor is that the trophoblasts which invade the lumen and straddle on the endothelial cells. The prevailing view of this endovascular trophoblast was first addressed by Hamilton and Boyd in 1965 in *Nature*. After reviewing numerous sections of placental bed biopsy, he concluded that cells in the lumen of spiral arteries which responsible for

vascular remodeling is the progeny of fetal cytotrophoblast. Moreover, this specialized cytotrophoblast is not from directly invading of the interstitial trophoblast, rather, should be from the trophoblast in the basal plate and migrating up to transform the spiral arteries. However, it arises some important queries to be answered. First, if the cytotrophoblast in the interstitial area does not invade the spiral artery directly, what is the purpose for its journey across the long distance area, from tips of cell column, pass by the decidual cells, and finally to the muscular area of the uterus? Second, what factor(s) is (are) the key driving force for these EVTs within the vessel lumen to against the arterial pressure to migrate up the spiral artery?

The interstitial cytotrophoblast once was thought to play a role in the immuno- regulation of embryo implantation. Nevertheless, the actual function of interstitial trophoblast is never elucidated. Recently, authors found these interstitial trophoblasts, after detaching the cell columns, usually colonize around the spiral artery. Therefore the authors presume that these interstitial trophoblasts may probably play a role in the transformation of spiral arteries, possibly priming some events, then the endovascular trophoblasts receive the signals and migrate up to the maternal uterine circulation. In fact, the invasion of interstitial trophoblast is also deeper than endovascular trophoblast: reaching a peak by the end of the first trimester and declined rapidly thereafter, the second wave in around 16 weeks and may deep into the myometrium.

In the first year of this project, we collect the sample from the decidua from the first-trimester D&C specimen, and also collect the placental bed biopsy form normal and preeclampsia third-trimester pregnancies during Cesarean section. We examine the interstitial trophoblasts in situ by immunohistocytochemistry, confocal microscopy, scanning electromicroscopy, transmission electromicroscopy. The aims of these examination are to understand the interrelationship of the trophoblast (interstitial, endovascular, multinucleated giant cells) and the implantation microenvirionment. The electromicroscopy may provide valuable information for the ultrastructure changes of trophoblast during implantation. Besides, we start the in vitro vulture and isolation of the trophoblast for the use of second year project.

Keywords: invasive trophoblast, implantation, vascular remodeling, human, cytokines.

二、緣由與目的

Successful human placentation depends on adequate transformation of the uteroplacental circulation by extravillous trophoblast (EVT) proliferation, migration, and invasion into the maternal decidua. This process rises to a peak by the end of the first trimester and declined rapidly thereafter. Human placental development depends critically on the differentiation of the placenta's specialized epithelial cells, termed cytotrophoblast. Two differentiation pathways exist.. In one, cytotrophoblast remain in the fetal compartment and fuse to form multinucleate syncytiotrophoblasts that cover the floating chorionic villi, whihch were direct contact with maternal blood in the intervillous space and, perform nutrient and gas exchange for the fetus. In the second pathway, a subset of cytotrophoblasts in anchoring villi aggregate into cell columns that attach to the uterine wall. From there, cytotrophoblast (CTB) invade the uterine wall and (interstitial invasion) and the decidual vessels (endovascular invasion) as far as the first third of the myometrium. As a net result, oxygenated maternal blood can thus largely flow into the intervillous space and provide nutrient for the fetus.

In normal pregnancy, CTB invade arteries more deeply than veins. Cells that are participating in endovascular invasion have two types of interactions with maternal arterioles. In the first type of interaction, large aggregates of these fetal cells are found primarily inside the vessel lumen. These aggregate can either lie adjacent to the apical surface of the resident endothelium or replace it such that they appear directly attached to the vessel wall. In the second type interaction, CTB are found within the vessel wall rather in the lumen. In this situation, they colonize the smooth muscle layer of vessel wall and lie adjacent to the endothelium. These two types of interactions may be representatives of the progressive stages in a single process, or indicative of different strategies by which CTB accomplish endovascular invasion. In either case, the stage in which fetal CTB cohabit with maternal endothelium in the spiral arterioles

is transient. By late second trimester, these vessels are lined exclusively by endometrial or the superficial portions of their myometrial segments. The specific purpose of this project is designed for the identifying the driving force of these EVTs and the whole process of the spiral transformation.

三、研究方法

I. SAMPLE COLLECTION: Chorionic villi and deciduas of the first-trimester pregnancy will be obtained from women undergoing elective termination due to blighted ovum or other psycosocial problems. Third-trimester specimen will be obtained during cesarean section at term.

II. IMMUNOHISTOCYTOCHEMISTRY

Tissue fixation, and immunocytochemistry

The tissues are fixed/rinsed/blocking according to the standard condition described *(Cell, Volume II: Subcellular localization of genes and their products, Spector DL et al., 1998 Cold Spring Harbor Laboratory Express).*

1. Tissues sections (5μm) are dewaxed and rehydrated conventionally.
2. Quenching of endogenous peroxidase is achieved by incubation with 0.3% hydrogen peroxide in methanol for 30 mins at room temperature.
3. All tissue sections are exposed to a non-immuned block with normal rabbit serum for 30 minutes at room temperature.
4. Incubation with the primary antibodies is carried out at 4°C overnight with various dilutions for the specific antibodies.
5. Thereafter tissues sections are labeled with an avidin-biotin-peroxidase detection system Vectastain (Vector Lab, Burlington VT, USA).
6. Each step is followed by a meticulous washing with PBS.
7. Finally, 3,3'-diaminobenzidine is used as chromogen.
8. Counterstaining was performed with hematoxylin.

For cryostat section (8-10μm), the steps are the same except skip the procedures of dewaxing and dehydration.

Antibodies

Markers for cytotrophoblasts

In chorionic villi and column portion of anchoring villi, cytokeratins are the most useful markers. Anti-pancytokeratins can stain syncytiotrophoblast, cytptrophoblast (stem cell), and also the invasive interstitial trophoblast. Nonetheless, the glandular epithelium, which thereotically regresses after 10 weeks' gestation, will be stained positive for anti-pancytokeratin. The architecture of glandular epithelium loos like transformed spiral artery with endovascular trophoblast in the lumen. We use CD56 (NCAM) as an immunomarker for the differentiation between endovascular trophoblast ($CD56^+$) and glandular epithelium ($CD56^-$).

Markers for smooth muscles component of the decidual vessels

We use phalloidin to recognize all the F-actin components in the placental bed biopsy. Phalloidin is a non-specific actin-binding toxin derived from mushroom. Either α, β, and γ forms of actin will be recognized by phalloidin. This will help to localize the decidual vessels (either vein or artery). We also use CD31 (PECAM) as a marker to localize the endothelial cells in the lumen of spiral artery.

Markers for cytokines that are putatively essential for implantation

We use a panel of antibodies to cytokines and their receptors that we intend to test the roles in the embryonic implantation.

Immunostaining

We use fluorescein-conjugated anti-mouse IgG as secondary antibodies to label the monoclonal antibodies, and rhodamine-antimouse IgG as seconday antibodies to label the polyclonal antibodies. Other fixation, washing, incubation techiniques are followed the standard protocols (see *Cells: a laboratory manual, by Spector DL, et al., Volume 3 Subcellular location of genes and their products*).

III. Transmission electromicroscopy (TEM)

IV. Scanning electromicroscopy (SEM)

V. CONFOCAL MICROSCOPY

Tissues sections (30μm) are fixed and stained in the standard processes of immunohistocytochemistry. The

post-stained slides will be examined using Zeiss confocal microscope (LSM 510). Optical sections will be collected at 0.5 micrometer. Steps through individual cell nuclei for analysis. Distribution of EGFP will be localized on the different optical sections.

VI. ISOLATION AND CULTURE THE TROPHBLAST FROM THE FIRST TRIMESTER PLACENTA

四、結果與討論

The followings are illustrations of our preliminary result for the first year project.

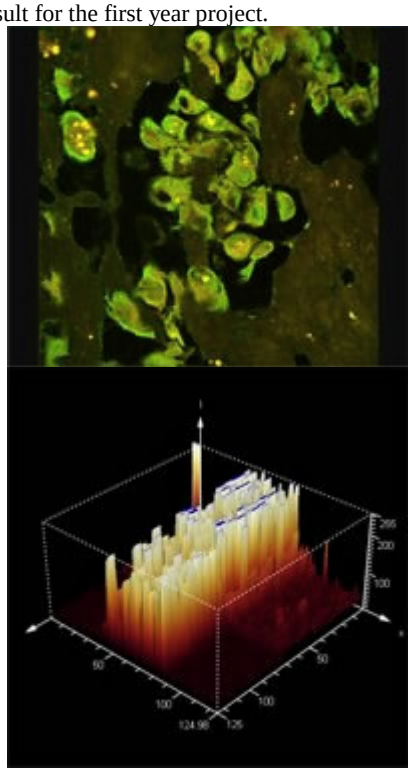


Fig.1 Confocal laser microscopy of placental bed biopsy of term pregnancy demonstrated the mature stellate-shape trophoblast in the Nitabuch layer.

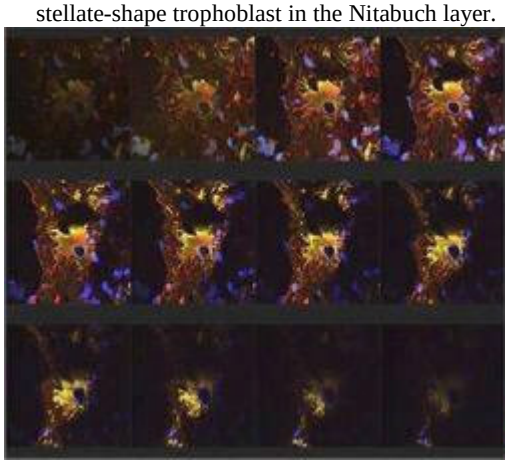


Fig.2 Confocal laser microscopy of a myometrium specimen from a 10-week gestation uterus (hysterectomy due to CIN III). It demonstrated stellate-shape trophoblast invading the vascular lumen of a spiral artery.

Fig.3 Confocal laser microscopy of a 1st passage cultured trophoblast from the first-trimester placental villi.

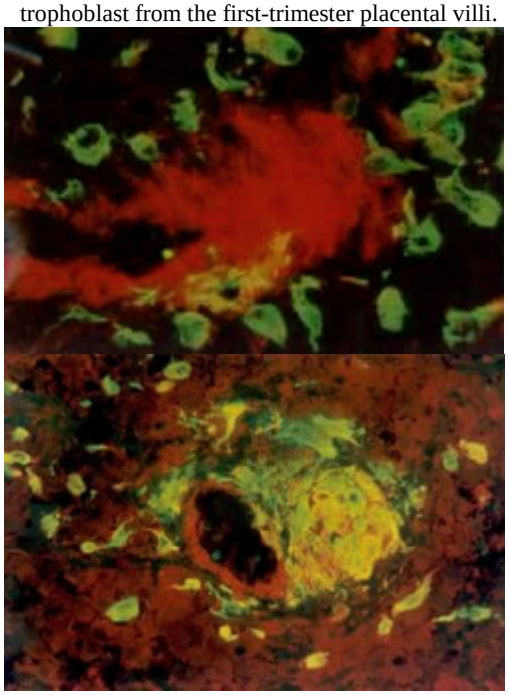
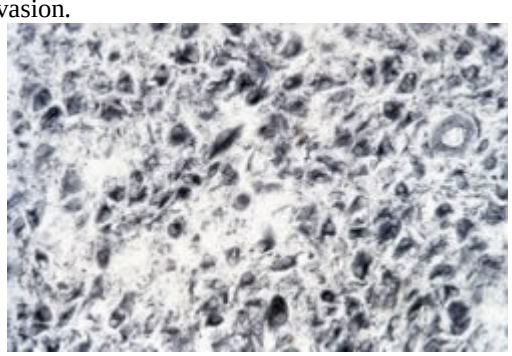


Fig. 4 Double immuofluorescence microscopy demonstrating the interstital trophoblast attack the spiral artery before the endovascular trophoblast invasion.



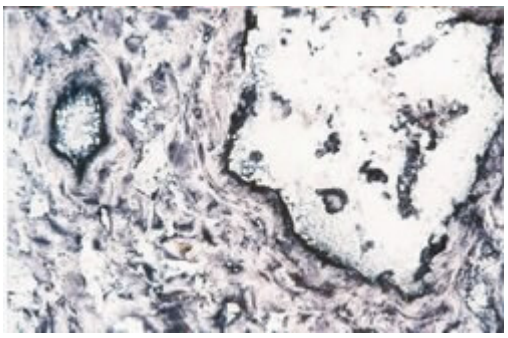


Fig. 5 Alkaline phasphotase conjugated with transforming growth factor β type I (upper) and type II (lower) receptors immunostaining. The section was located in the deep deciduas and decidual artery were also seen. Positive stainings were evident in the decidual cells and endothelial cells of spiral arteries.

We found the invasion of extravillous trophoblast is associated with downregulation of the receptor (both type I and II) of transforming growth factor β (TβRI and II) when these trophoblasts detach cell columns. The trophoblast invasion into deep decidua is also associated with the upregulation of TβRs of implntation environment- deep decidual tissue and myometrium. These changes occur with switching of integrins repertories. Moreover, the physiological adaptation of spiral artery are also paradoxically associated with the upregulation of TβRs of extravillous trophoblasts around the perivascular space, indicating the TGFβ is one of the key regulator of trophopblast invasion. The switching of TGFβ receptors of trophoblast is also associated the phenotype change: from unipolar motile form to multipolar, stellate shape trophoblast. It also indicates TGFβ might affect the morphology and function in the process of spiral artery remodeling.

We concluded the trophoblast invasion is determined both the intrinsic factors and also by paracrine factors, such as the cytokines producing by deciduas and myometrium. The physiological changes of spiral artery during pregnancy is a unique process related to spatial and temporal changes of growth factor expression and itself phenotypes.

五、計畫成果自評

The results obtained from current project were exciting.. Not only we found the role of TGFβ in implantation and associated with integrins repertoire switching, but also identified the important event in the process of spiral remodeling.

Besides, the establishment of first-trimester trophoblast culture was already successful. It will provide the materials for the sophisticated culture system that was designed for confirmation of our novel findings in the first year project.

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