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黃體素接受體 A 和 B 型在卵巢癌的角色

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黃體素接受體 A 和 B 型在卵巢癌的角色(NSC 91－2314－B－002－383)

中文摘要: 卵巢癌細胞株 OVTW59 含有黃體接收器（PR）A 及 B，PRA 和細胞侵入有關。以 PRA 及 PRB 進行細胞轉殖，OVTW59 的活性 FAK 大量表現，但細胞游走能力下降，尤其以 PRA 為甚。

關鍵詞：卵巢癌細胞株、黃體接收器、侵入、細胞轉殖、FAK、遊走

英文摘要: Progesterone receptors A and B were found in the ovarian carcinoma cell line OVTW59-P0 and PRA is associated with cancer invasiveness. Transfection of PRA and B into OVTW59 cell lines resulted in increased active FAK, but decreased in cancer migration, especially in PRA then PRB.

Keywords: Ovarian adenocarcinoma cell line, progesterone receptor, invasion, transfection, FAK, migration.

報告內容:

前言: Function of progesterone receptors A and B in the cancer progression and metastasis of ovarian carcinoma is unknown.

計畫目的：To study the role of progesterone receptors in ovarian carcinoma invasion by using transfection assay in ovarian carcinoma cell lines with different invasive capabilities.

文獻探討:

Most ovarian tumors were reported to contain estrogen and progesterone receptors (37%-69% for ER and 20%-68% for PR) (1,2) and tumors containing ER and PR tend to be more responsive to therapy than those that are ER and PR negative (3,4). More specifically, an increase in PR was correlated with increased survival (2-4). Two distinct forms of progesterone receptors in human (h), the hPR-A, 94kDa and hPR-B, 114 kDa, are produced from a single gene and are translated under the contrast of distinct promoter (5). In most cell contents, hPR-B functions as a transcriptional activator of progesterone-response genes, whereas hPR-A functions as a transcriptional inhibitor of all steroid hormone receptors (6). Both PR-A and PR-B are co-expressed in the same target cells in human (7), and their relative expression, where it has been examined, generally is close to unity (8). Most ovarian carcinomas are found to be PR-B predominant (10 to 20 fold higher) (9), and lower PR-B level (by immunohistochemical staining and RT-PCR) is found to be an independent poor prognostic factor (9). Therefore, down regulation or modification of PR-B to lower its expression may be one of the ways that carcinoma cells processed to gain greater metastatic behavior.

In this study, we studied the hPR-A and hPR-B in our newly established endometrioid adenocarcinoma cell line OVTW59 and its highly invasive sub-line as

to investigate the role of progesterone receptors in the role of cancer invasiveness in ovarian cancer.

材料及研究方法:

Materials

Ovarian carcinoma cell line OVTW 59 (P0) and its highly invasive sublines,P4 were cultured in Dulbeecco's modified Eagle's medium (DMEM) solution with 5% heat inactivated fetal calf serum (FCS). A human breast carcinoma cell line ZR-75-1 obtained form Bioresources Collection and Research Center were cultured in RPMI-1640 solution in 10% FCS.

MTT assay for progesterone, estrogen and antiprogestin (RU486) effect:

Exponentially growth Po and P4 cells were seeded into 96-well-flat bottom tissue culture plates (2000 cells/well in 0.1 ml) overnight, and progesterone 2 ug/ml, serial concentrations of RU486 (3.7, 5,10,15 ug/ml) were added to individual wells and cultured for 2 days. Cell survivals were analyzed by MTT assay. MTT (0.5ml, 2 mg/ml) was added to each well for 2.5 hours. Supernatants were aspired and the blue crystals formed were dissolved in 0.15 ml Dimethyl sulfoxide (DMSO) solution and assessed at 540 nm by an enzyme-linked immunosorbent assay reader. All data points represent the average of triplicate wells.

Immunostaining of the progesterone receptors in culture cells (10,11)

Cell were seeded at 60% confluence on cover slides.10ug/ml of progesterone, 50 ug/ml of estrogen, or 10ug/ml of RU-486 were added for 24 hours and then the slides were fixed with 3% formaldehyde (EM grade) for 10 minutes and then immunostained using the avidin-biotin (ABC) immunoperoxidase technique. Primary antibodies of progesterone receptors (Dako, MA, clone 1A6, diluted 1:10; or Santa Cruz, MA, clone AB-52, diluted 1:50; or Neomarker, MA, clone hPRa2+hPRa3, diluted 1:100, for PR-A and PR-B), (Neomarker, MA, clone hPRa2, diluted 1:100, specific for PR-B), (Neomarker MA, clone hPRa7, diluted 1:100, specific for PR-A) were incubated overnight at 4℃ and biotin labeled secondary antibody for 1 hour. Reaction were amplified by ABC (avidin-biotin-peroxidase complex) and color developed by 0.05% DAB (3,3'-diaminobenzidine-4 HCL in 0.05M Tris-HCL, pH 7.2) and counterstained with 0.01% OsO_4.

Semiquantitative Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA of P0 and P4 cells were homogenized and extracted using Ultraspec RNA isolation solution. RNA pellet were dissolved in DEPC-H2O and the concentration determined by spectrophotometry. 2 ug of total RNA were reverse

transcripted and 1-2 ul of RT production were for PCR reactions containing 10 mM dNTPs, 10 mM Tris-HCL, pH 8.3, 50 mM KCL, 0.1% Triton X-100, 1.5mM MgCl2, 2ug Tag polymerase (Promega) and 0.5 uM primers of PRB (sense: ACAGAATTCATGACTGAGCTGAAGGCAAAGGGT, antisense: ACAAGATCTCAAACAGGCACCAAGAGCTGGTGA) or primer common to PRA and B (sense:ACAGAATTCATGAGCCGGTCCGGGTGCAAG, antisense: ACAAGATCTCCACCCCAGAGCCCGAGGTTT) and S26 (20 ng) as positive internal control (sense: CCGTGCCTCCAAGATGACAAAG, antisense: ACTCAGCTCCTTACATGGGCTT) in 25 ul. After an initial denaturation step at 94℃, 28, 30, 32 and 34 cycles per PCRs were carried out on a DNA thermal cycler (PTC-200 DNA Engine, MJ Research Inc, USA) under the following conditions: 1 min denaturation at 94℃, 1 min annealing at 58℃, and a 30 second extension at 72℃.

Immunoblotting of PRs

Cells were harvested and protein extracted by adding 200μl of buffer C (20mM HEPES at pH 7.9, 0.4N NaCl, 1mM EGTA) , and 2X protease inhibitor (0.1MPMSF, 1MDTT, 1mg/ml PepA, 1Mleupeptin, 0.2%Triton-X100), centrifuged and followed by equal volume of buffer A (0.1mM EDTA, 10mM HEPES at pH 7.9, 10mM of filtered KCl, 0.1mM EGTA).Alternatively, cytopasmic and nuclear protein were extracted by NE-PER TM according to manufacture's illustration. Protein concentrations were determined by the Bio-Rad protein assay. 50μg per lane were electrophoretically separated on a 6% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) in the sample buffer, transferred onto PVDF membrane using the MilliBlot-SDS semi-dry electroblotting system (Millipore), washed and blocked in blocking solution (5% nonfat milk, 0.9 % NaCl, 0.1% tween-20, 10mM tris-HCL (pH 7.4), 0.002% antifoam A) for 2 hours. Primary antibodies, such as progesterone receptors (NeoMarkers, CA, clone hPRa2 + hPRa3, diluted 1:500), FAK (Santa Cruz, H-1, diluted 1:200), PYK2 (Santa Cruz, N-19, diluted 1:200), tubulin (NeoMarkers, CA, DM1A + DM1B, diluted at 1:1000) and neucleolin (from Professor Lee Sheng-Chung from the Institute of Molecular Medicine, National Taiwan University, diluted at 1:1000, as internal control) were reacted at 4°C overnight, blocked, probed with biotinylated second antibody for 1 hour, reacted with streptavidin-HRP 30 for minutes and exposed for film development.

Transient transfection

Transfection was carried out using the lipofectamin 2000 (Life Technologies). The constitutive expression vector PRA (pOP13 hPRA plasmid provided by Dr CL

Clarke from University of Sydney) and PRB encoding the lac repressors (provided by Dr P. Chambon from INSERM, Universite Louis Pasteur, Paris, France) was used. 4 x 10^5 P0 and P4 cells were seeded into six-well tissue culture plates before the day of transfection in 2 ml culture medium. Cells were incubated with 6μl of lipofectamin 2000 in 1ml serum free medium containing 2μg of plasmid. Incubation of the cells with transfection medium will be continued for approximately 6 h at 37 C in 10% CO2. After transfection, the culture medium was changed to medium containing 5% FCS for another 24 hours and cells were assayed for protein expression by Western blotting and for further biological experiments.

Scratch (Wounding) Assay

Confluent monolayer of cells were transiently transfected and maintained in culture medium for 24 hours. A 200-μl plastic pipette tip was used to scratch the monolayers. The cells were then cultured in medium for an additional 17 hours, and photographed under an inverted phase contrast microscope.

結果 (Results)

1. OVTW59 cell lines were mildly stimulated by progesterone, and these progesterone effects were totally inhibited by RU-486. Po cells showed mild stimulatory effect at low RU-486 level and inhibitory effects at higher RU-486 levels (Fig 1). However, the P4 cells showed only inhibitory effects of RU-486 (Fig 2).

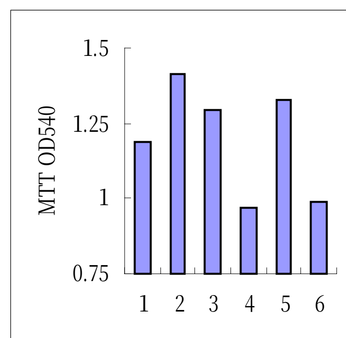

Fig 1. In vitro growth effect of P0 cells using MTT assay showing mild stimulation effects of progesterone (bar 2) and low dose RU-486 (bar 3). Effect of progesterone was completely blocked by RU-486 in the combination of two drugs (bars 5 and 6). 1: Control, 2: progesterone at 2 ug/ml, 3: RU-486 at 7.5 ug/ml, 4: RU-486 at 15 ug/ml, 5: progesterone at 2 ug/ml + RU-486 at 7.5 ug/ml, 6: progesterone at 2 ug/ml + RU-486 at 15 ug/ml,

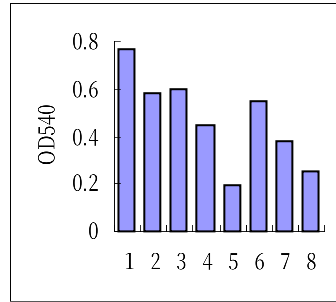

Fig 2. In vitro growth effect of P4 cells using MTT assay showing mild inhibition effect of progesterone and RU-486 (even at low dose of RU-486). Effect of progesterone was completely blocked by RU-486. 1: Control, 2: progesterone at 2 ug/ml, 3: RU-486 at 3.7 ug/ml, 4: RU-486 at 7.5 ug/ml, 5: RU-486 at 15 ug/ml, 6: progesterone at 2 ug/ml + RU-486 at 3.7 ug/ml, 7: progesterone at 2 ug/ml + RU-486 at 7.5 ug/ml, 8: progesterone at 2 ug/ml + RU-486 at 15 ug/ml,

2. Immunohistochemical staining of PRA and PRB in OVTW59 P0 and P4 cells.
   Both hPRA and hPRB were mainly localized in the cytoplasms of P0 and P4 cells, especially at the perinulcear areas. hPRA was found localized in the nucleus in some cells. Pharmacological treatments (progesterone, estrogen and RU-486) of Po and P4 cells did not affect the distribution of PRA and PRB.

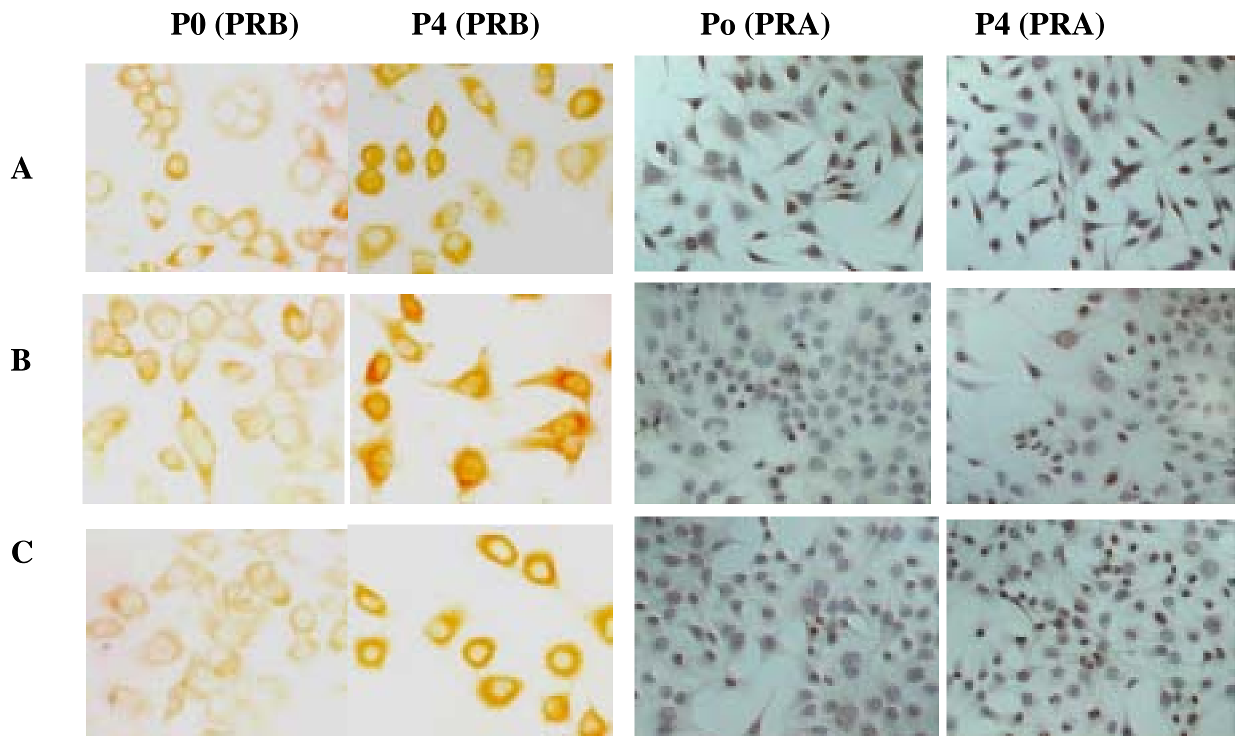

Fig 3. Immunohistostaining of PR-A and PR-B in OVTW 59 P0 and P4 cells after pharmacological treatment of progesterone (A, 10 ug/ml), estrogen (B, 50ug/ml) and RU-486 (C, 10 ug/ml).

3. Messenger RNA of PR-A and PR-B in P0 and P4 cells using semi-quantitative RT-PCR.

   We failed to detect the PR-B mRNA levels by RT-PCR after trying 3 different pairs of documented PR-B specific primers. By using SS26 as an internal control, we found P4 cells expressing higher amounts of PRA/B then P0 cells.

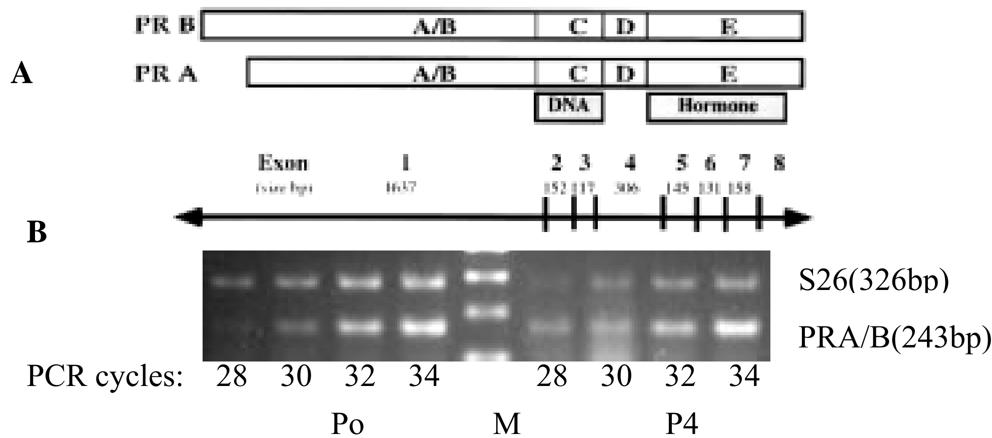

Fig 4. A. Funtional regions of hPRA and hPRB. B. Semi-quantitative RT-PCR showing PR-A/B mRNA expression in the P0 and P4 cells. P4 cells showed greater amount of hPR A/B then P0 cells when comparing with S26 (Internal control) at PCR 28, 30, 32, 34 cycles.

4. Western blotting of PRA and PRB expressions in P0 and P4 cells.

   More PRB was detected in P0 then P4 cells. PRA were localized in the nucleus as well as cytoplasm.

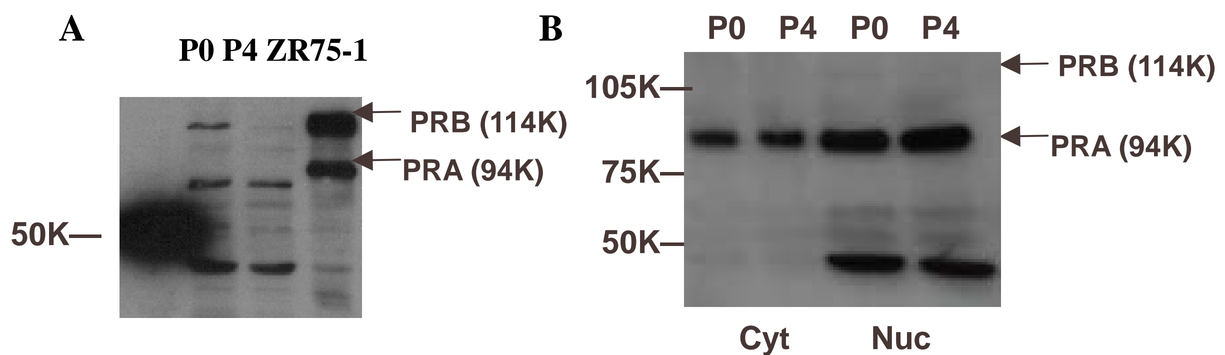

Fig 5 Western blotting to show the PR-A and P-B protein expression of P0 and P4 cells in the whole cell lysate (A) and in the cytoplasmic (Cyt) and nuclear (Nuc) protions (B).

5. PRA and PRB transfection and migration assay.

   PRA and B were transfected into OVTW59 P0 and P4 cells and the results of

transfection were analyzed with Western blotting and migration assay.

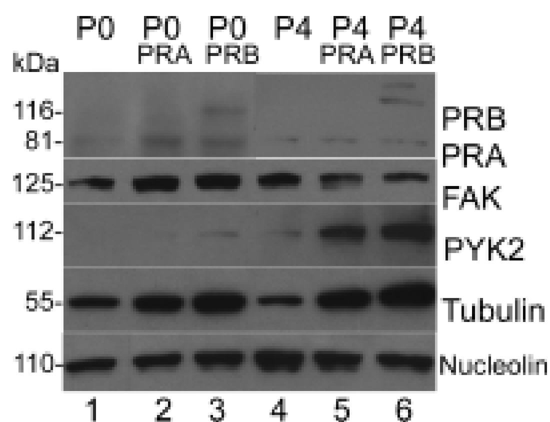

Fig 6. Western blotting to show PRA, PRB,FAK, PYK2 (FAK-Phosphate), tubulin and neocleolin expression after PRA (lane 2,5) and PRB transfection (lane 3 and 6).

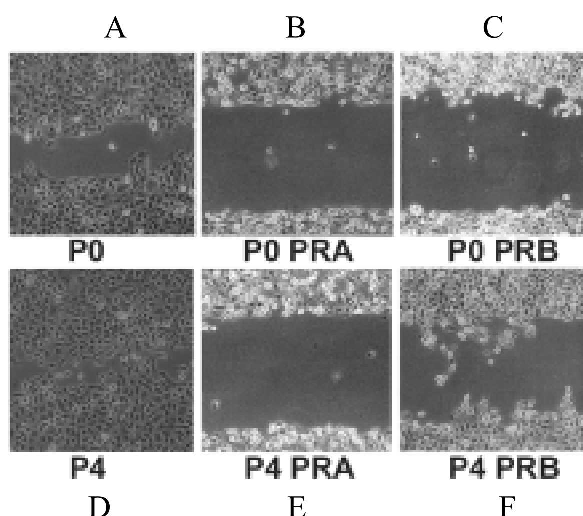

Fig 7. Scratch (wounding) assay showing higher migration capability of P4 then P0 cells. P4 cells showed significant cell migration at 48 hours (D) then P0 cells (A). The migration capability was remarkable reduced after PRA (B,E) then PRB (C,F) transfection.

討論 (Discussion)

We found presence of progesterone receptors (PR) A and B in cell line OVTW59, and low PRB and high PRA in the highly invasive subline P4, similar to the hypothesis that PRA is associated with poor prognosis (9). The in-vitro assay showed mild growth stimulation effect of progesterone in P0, and inhibition in P4 cells, corresponding to the report that PR-B was transcriptional active on the progesterone responsive element-containing promoters (5,6,12) and PR-A a transcriptional inhibitor of other steroid hormone receptors, including ER and PRB

(6,12,13), and the report that the agonistic activity of antiprogestins was specific to the presence of PRB (14,15).

By immunohistochemical staining, we found cytoplasmic localization of PRB and nuclear localization of PRA unresponsive to hormonal (estrogen, progesterone and RU-486) stimulation. This suggests PRA to be constitutively active in OVTW59.

We found increase of P4 migration capability with increased active FAK. This result is consistent with the report that FAK regulates the biological process of cancer pathogenesis (16). However, elevation of active FAK after PRA and B transfection in OVTW59 P0 and P4 cells did not show increase of migration capability. And indeed, inhibition of migration capability in P4 cells after PRA transfection. This suggests: 1. Amount of PRA is important in controlling cell migration 2. PRA might induce cell growth inhibition or rounding-up of cells (17,18) that cause reduction of cell migration. 3, Endogenous progesterone is important for cancer cell migration, since OVTW59 did not secrete progesterone and the endogenous PRA in OVTW59 are constitutively active (ie, progesterone is not necessary for its nuclear translocation), while progesterone is required for the transfected PRA and B to be active. In addition, we found incomplete inhibition of P4 cell migration after PRB transfection. Further study is necessary to clarify PRA and B in the mechanism of cell migration.

參考文獻 (References)

1. Ahlgren JD, Ellison NM, Gottlieb RJ, Laluna F, Lokich JJ, Sinclair PR, Ueno W, Wampler GL, Yeung KY, Alt D, Fryer JG. Hormonal palliation of chemoresistant ovarian cancer: three consecutive phase II trial of the mid-atlantic oncology program. J Clin Oncol 1993;111957-68.
2. Langdon SP, Gabra H, Bartlett JM, Rabiaz GJ, Hawkins RA, Tesdale AL, Ritchie AA, Miller WR, Smyth JF. Functionality of the progesterone receptor in ovarian cancer and its regulation by estrogen. Clin Can Res 1998;4:2245-51.
3. Münstedt K, Steen J, Knauf AG, Buch T, von Georgi R, Franke FE. Steriod hormone receptors and long term survival in invasive ovarian cancer. Cancer 2000;89:1783-91.
4. Hempling RE, Piver MS, Eltabbakh GH, Recio FO. Progesterone receptor status is a significant prognostic variable of progesterone-free survival in advanced epithelial ovarian cancer. Am J Clin Onc 1998;21:447-51.
5. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P. Two distinct estrogen-regulated promoters generate transcripts encoding the two functional different human progesterone receptor forms A and B. EMBO J 1990;126:891-8.
6. Wen DX, Xu YF, Mais DE, Goldman ME, McDonnell DP. The A and B isoforms

of the human progesterone receptor operated through distinct signaling pathways within target cells. Mol Cell Biol 1994;14:8356-64.

7. Mote PA, Balleine RL, McGowan EM, Clarke CL. Co-localization of progesterone receptors A and B by dual immunofluorescent histocehmistry in human endometrium during the menstrual cycle. J Clin Endocrinol Metab 1999; 84:2963-71.
8. Pratt WB, Toft DO. Steriod receptor interactions with heat shock protein and immunophilin chaperones. End Rev 1997;18:306-60.
9. Akahira J, Inoue T, Suzki T, Ito K, Konno R, Sato S, Moriya T, Okamura K, Yajima A, Sasano H. Progesterone receptors isoform A and B in human epithelial ovarian carcinoma: immunohistochemical and RT-PCR studies. Br J Cancer 2000:83:1488-94.
10. Lin CT, Chan WY, Chen W, Shew JY. Nasopharyngeal carcinoma and retinoblastoma gene expression. Lab Invest 1992;67:56-70
11. Lin CT, Dee AN, Chen W, Chan WY. Association of Estein-Barr virus human papilloma virus, and cytomegalovirus with nine nasopharyngeal carcinoma cell lines. Lab Invest 1994;71:731-6.
12. Giangrande PH, Pollio G, McDonnell DP. Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. J Biol Chem 1997;272:32889-900.
13. Kraus WL, Weis KE, Katzenellenbogen BS. Inhibitory cross-talk between steroid hormone receptors: differential targeting of estrogen receptor in the repression of its transcriptional activity by agonist- and antagonist-occupied progestin receptors. Mol Cell Biol 1995;15:1847-57.
14. Meyer ME, Pornon A, Ji J, Bocquel MT, Chambon P, Gronemeyer H. Agonist and antagonistic activities of RU486 on the function of the human progesterone receptor. EMBO J 1990;9:3923-32.
15. Sartorius CA, Tung L, Takimoto GS, Horwitz KB. Antagonist-occupied human progesterone receptors bound to DNA are functionally switched to transcriptional agonists by cAMP. J Biol Chem 1993;268:9262-6.
16. Gabarra-Niecko G, Schaller MD, Dunty JM. FAK regulates biological processes important for the pathogenesis of cancer. Cancer Meta Rev 2003;22:359-74.
17. McGowan EM, Weinberger RP, Graham JD, Hill HA, Hughes JAI, O'Neill GM, Clarke CL. Cytoskeketal responsiveness to progestins is dependent of progesterone receptor A levels. J Mol Endocrinol 2003;31:241-53.
18. Lin VCL, Ng EHN, Aw SE. Tan MGK, Ng EHL, Bay BH. Progesterone induces focal adhesion in breast cancer cells MDA-MB-231 transfected with progesterone receptor complementary DNA. Mol Endocrinol 2000;14:348-58.

計畫成果自評:

1. PRA and PRB transfection showed markedly elevation of FAK and its phosphorylated form. Since FAK was reported to play a pivotal role in the initiation and progression of tumor formation and metastasis. This result indicated increasing in cancer phenotype after PRA and PRB transfection. However, we contradictory showed decreased in cell migration, especially in PRA then PRB transfection. Several investigators have reported rounding-up of cells after PRA and PRB transfection and reduction of cell proliferation after transfection. We need to further clarify this.
2. Stable PRA and PRB transfection instead of transient transfection is better for result interpretation. Next, we need a control transfection in future study.
3. Further studies are now still carrying on, such as immunostaining to se the fuction of PRA and PRB after transfection, adding progesterone to see the effect of migration and invasion after PRA and PRB transfection; and the establishment of stable transfection cell lines.