出國報告(出國類別：進修)

產前遺傳學暨胎兒醫學

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摘要

I was very lucky to get the scholarship from the Department of Health, Executive Yuan in 2008 without which it was extremely difficult for me to afford such a high expense of tuition fee for postgraduate study in England. I attended a MSc course of UCL (University College London) in September 2011 which was in accordance with my clinical field. The full name of this postgraduate course is MSc of Prenatal Genetics and Fetal Medicine that is composed of taught courses for 9 months and a experimental project for 3 months. The taught courses were divided into 8 modules which were arranged in 3 terms. These modules are 1. Basic Genetics and Technology, 2. Gametogenesis, Preimplantation Development and IVF, 3. Genetic Mechanisms, 4. Medical Genetics, 5. Prenatal Diagnosis, 6. Organogenesis, 7. Fetal Medicine, 8. PGD (Preimplantational Genetic Diagnosis). One module was evaluated by booklet, another 3 modules were evaluated by essays and the other 4 modules were evaluated by examinations. The project I chose is “In-Utero Transplantation of Human Amniotic Fluid Stem Cells in Mice” and the data from the experiments were analysed in the thesis that was composed of around 10,000 words and was a requirement for this MSc degree.

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壹、目的

隨著超音波及產前遺傳診斷的進步，許多先天性胎兒異常及遺傳疾病已可於出生前被發現，但大多數疾病目前並無治療對策，故多數父母選擇人工流產以終止妊娠。若能利用基因醫學或幹細胞發展出治療方法，希能提供父母另一選擇。幹細胞治療亦可運用於再生醫學，治療神經、心臟、肝臟等器官疾患。

此次進修內容：羊水幹細胞研究、胎盤幹細胞研究、產前基因治療研究、產前幹細胞治療研究。

預期達成目標及效果：學習幹細胞醫學新知、學習基因治療醫學新知、學習羊水及胎盤幹細胞實驗方法、研究發展胎兒治療及再生醫學、並取得相關碩士學位。另外，提升本院研究風氣、結合本院分子生物研究室進行羊水及胎盤研究、將本院婦產科轉型為研究及醫療並重之科室、建立胎兒醫學之新觀念以提升本院婦產科醫療水準符合醫學中心要求，也是此行重要之目的。

I was born and educated in Taipei. After graduation, I've practiced Obs and Gyn for 16 years up to 2010. The more experienced I am clinically, the more confused I feel about the meaning of antenatal examination. Basicly what I've learned and what I could provide is just kind of "judgement" of fetuses. Judge them "normal" or "abnormal", "healthy" or "unhealthy", "reassuring" or "risky", then leaving the decision to parents for opting either acceptance or termination, without bringing any welfare to the fetuses.

It's extraordinarily refreshing to me to study fetal medicine in the UK. Although nowadays there are very few fetal therapies that are feasible and can be applied clinically, plenty of researches are going on and enormous efforts have been devoted in the UK from the perspective of fetal well-being. Although neither do fetuses in the UK have their own rights before delivery, both scientists and clinicians have been proclaiming many ethical considerations.

Stem cells are promising in regenerative medicine, and the patients may be benefited more if the treatment is given extremely early when the damage by the disease is not that deleterious. Hopefully, the combination of stem cells and fetal therapy may reveal its supremacy over postnatal therapy in the near future and contribute to both fetuses and parents.

貳、過程

Stem cells represent a certain type of cells that are characterized by their unique ability to replicate and differentiate into diverse range of specialized cells. Because of their high plasticity, stem cells are ideal to be used in repairing damaged tissues. According to different derivations, the human stem cells can be categorized into embryonic stem cells (ESCs), induced pluripotent stem cells (iPS cells) and adult stem cells.

Mesenchymal stem cells (MSCs) are the multipotent or even pluripotent stem cells that own the characteristics of multilineage differentiation and can be applied in regenerative medicine of different organs rather than hematological diseases alone. Due to the prevailing of prenatal screening and diagnosis, amniocentesis and chorionic villus sampling (CVS) are performed very often in maternal-fetal unities and the specimens obtained (amniotic fluid and placental tissue) not only facilitate kayrotyping but also provide alternative sources for retrieving the MSCs or other pluripotent stem cells.

Because the timing of amniocentesis and CVS are quite early in the whole gestational course, the stem cells retrieved from these 2 procedures are very promising in either fetal therapy or postnatal treatment of some congenital diseases.

C-Kit (CD117) is a cytokine receptor expressed in high level on hematopoietic stem cells, multipotent progenitors (MPP), and common myeloid progenitors (CMP). It binds to stem cell factors and plays a crucial role in cell survival, proliferation, and differentiation. My project is to evaluate and compare the migrating potential between human AF stem cells and human placental stem cells via C-Kit selection and intrauterine transplantation to fetal mice, and hopefully this xenogenic model may facilitate autogenic and allogenic model in the future.

With the enthusiasm for clinical application, we would like to explore the *in vivo* potential of fresh and cultured human AFS cells for engraftment and differentiation using IUT in mice, to demonstrate that IUT of human AFS cells in mice is feasible and to confirm that the fresh human AFS cells possess more haematopoietic potential than the cultured ones.

We designed 2 groups of IUT in mice including cultured human AFS cells and fresh human AFS cells. Human amniotic fluid from amniodrainage at 34 weeks was

recruited for the first group and 60,000 cultured human AFS cells were implanted to each fetal mouse on E14. Human amniotic fluid from amniodrainage at 35 weeks was recruited for the second group and 10,000 fresh human AFS cells were implanted to each fetal mouse on E14. The peripheral blood of recipient mice was analysed by flow cytometry at 4 weeks of age. The recipient mice were sacrificed at 6 weeks of age and 8 organs were dissected. The engraftment was detected by flow cytometry (FACS analysis), PCR and IF stain. Cells of bone marrow were also used for colony-forming cells (CFC) assays.

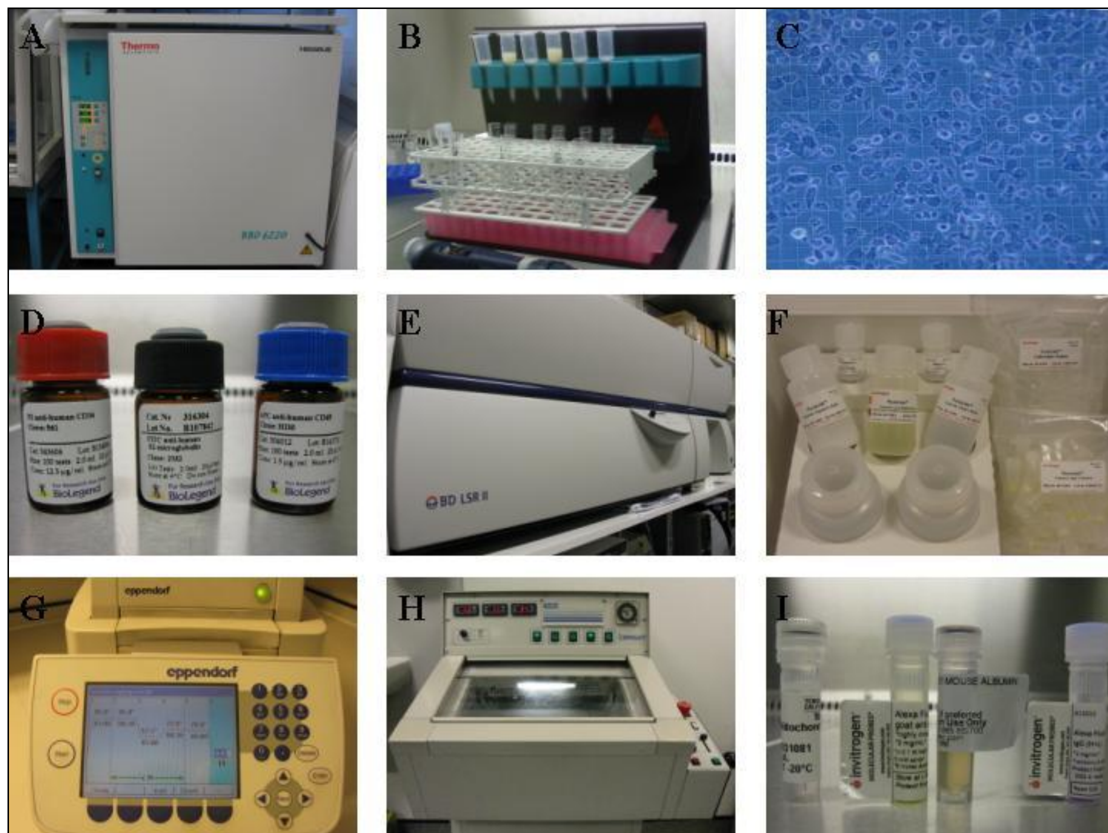

Fig. 1. The incubator for stem cells expansion (A). Magnetic-sorting column (B). Neubauer hemocytometer (C), the volume of 20x20 squares is 0.1μ l. 3 antibodies for our FACS analysis (D). BD FACSCalibur LSR II (E). Kits for DNA extraction (F). Eppendorf thermal cycler (G). Cryostat (H). 4 antibodies for our IF stain (I).

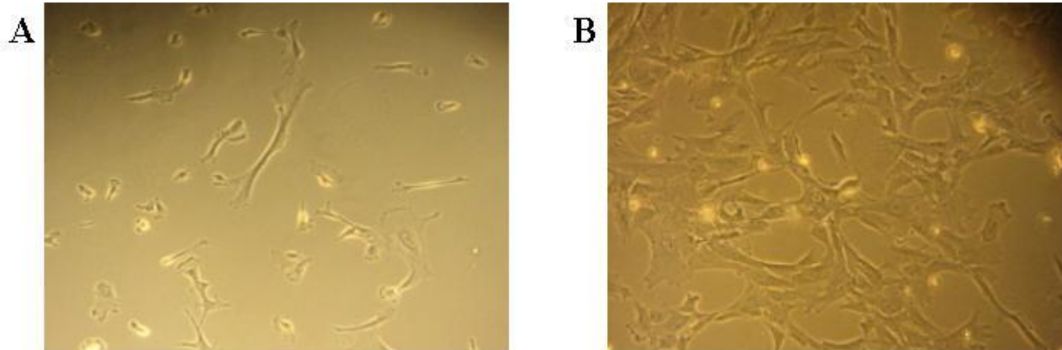

Fig. 2. Cultivation of human AFS cells. The AFS cells adherent to the plate (A). The confluent AFS cells before passage (B).

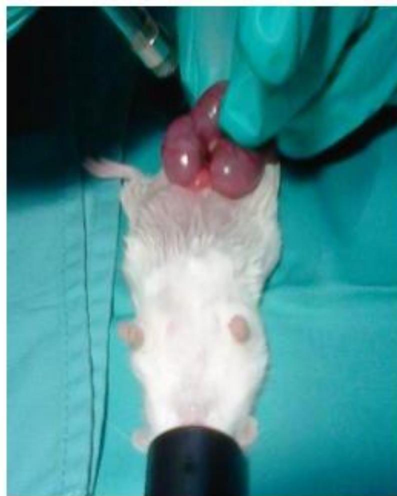

Fig. 3. IUT in fetal mice. Within the uterus each fetus was identified and human AFS cells were implanted to the peritoneal cavity beneath the fetal liver via microinjection.

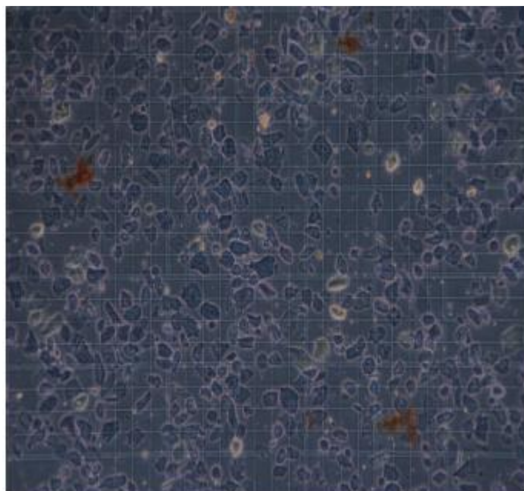

Fig. 4. The fresh amniocytes from the amniodrainage of gestational 35 weeks for the group of fresh frozen human AFS cells. The majority of cells on Neubauer haemocytometer by trypan blue exclusion assay were not viable.

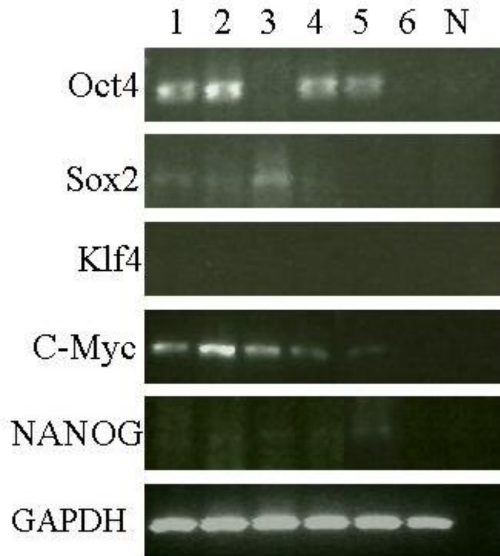

Fig. 5. RT-PCR of the extracted RNA from the stem cells of human amniotic fluid for characterisation. N: negative control. 1, 2, and 3 are from the AF for the group using cultured AFS cells. 4, 5, and 6 are from the AF for the group using fresh frozen AFS cells. 1 and 4 are unsorted cells. 2 and 5 are C-Kit(+) cells. 3 and 6 are C-Kit(-) cells. C-kit(+) cells from human amniotic fluid were named as human AFS cells.

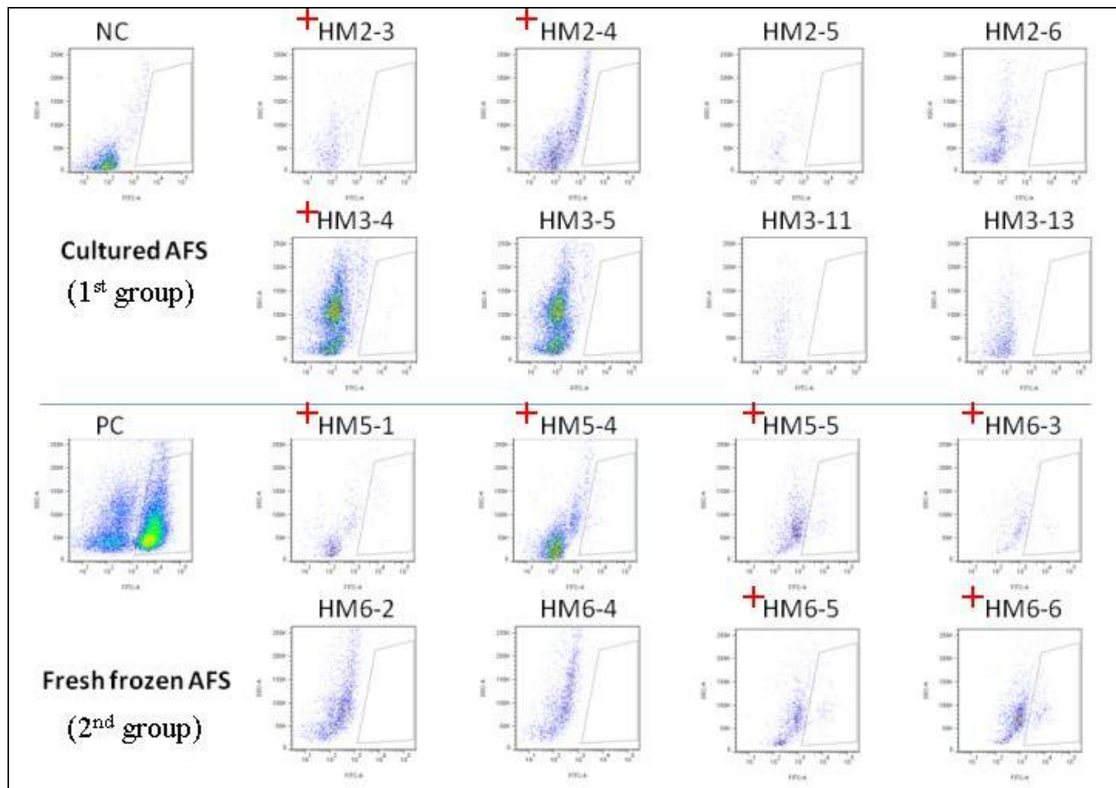

Fig. 6. FACS analysis in the blood of recipient mice at 6 weeks after birth. NC: negative control is the blood of a mouse without any procedure. PC: positive control is human blood. +: positive for human β2 microglobulin by analysis. In comparison with the 2nd group, of the positive recipient mice, the percentage of cells in positive channel is obviously less in the 1st group.

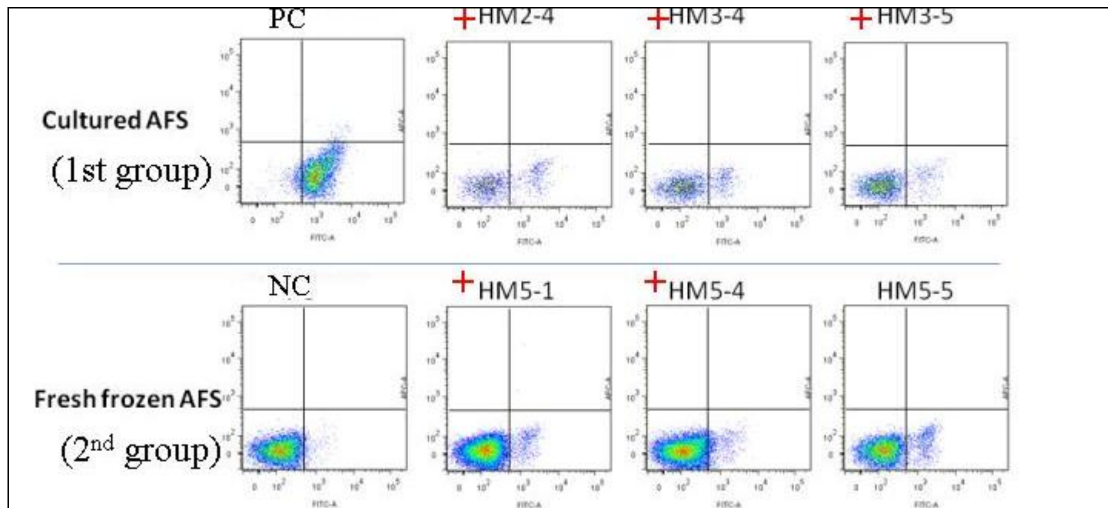

Fig. 7. FACS analysis in the liver of recipient mice at 6 weeks after birth. PC: positive control. NC: negative control. +: positive for human β2 microglobulin by analysis. In comparison with the 1st group, of the positive recipient mice, the percentage of cells in positive channel is greater in the 2nd group.

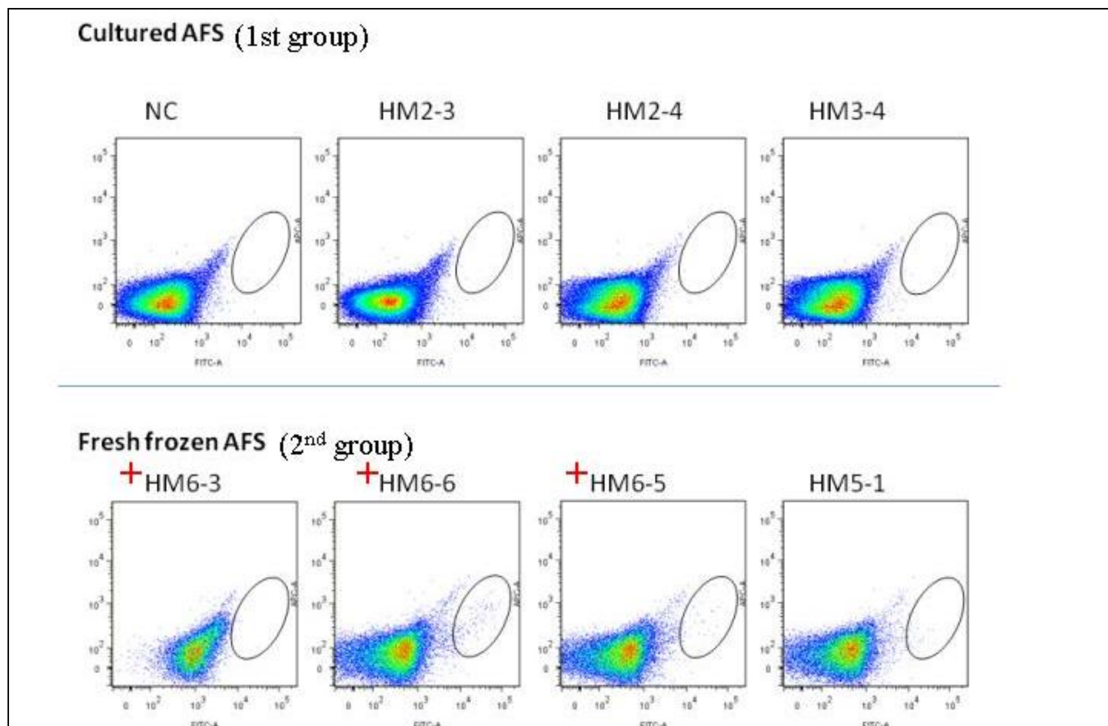

Fig. 8. FACS analysis in the spleen of recipient mice at 6 weeks after birth. NC: negative control. +: positive for human β2 microglobulin by analysis. The method of gating is different from Fig. 6 and Fig. 7. All the recipient mice in the 1st group were negative.

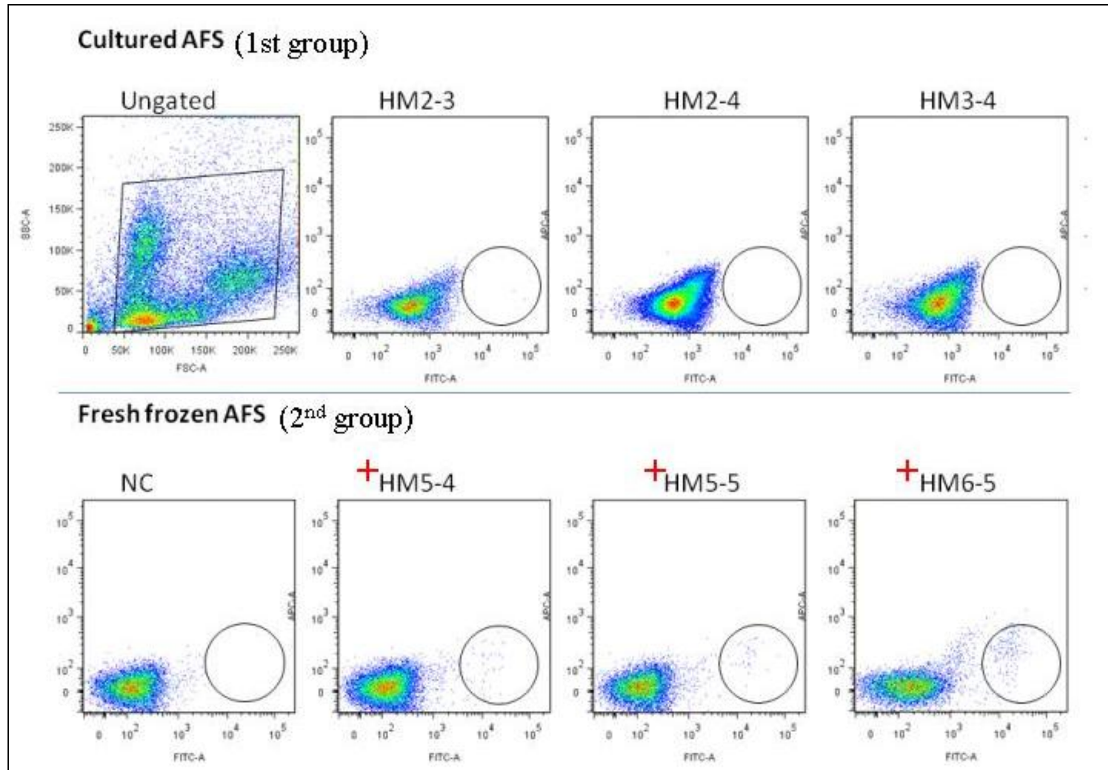

Fig. 9. FACS analysis in the bone marrow of recipient mice at 6 weeks after birth.
NC: negative control. +: positive for human β2 microglobulin by analysis. The method of gating is different from Fig. 6, Fig. 7 and Fig. 8. All the recipient mice in the 1st group were negative.

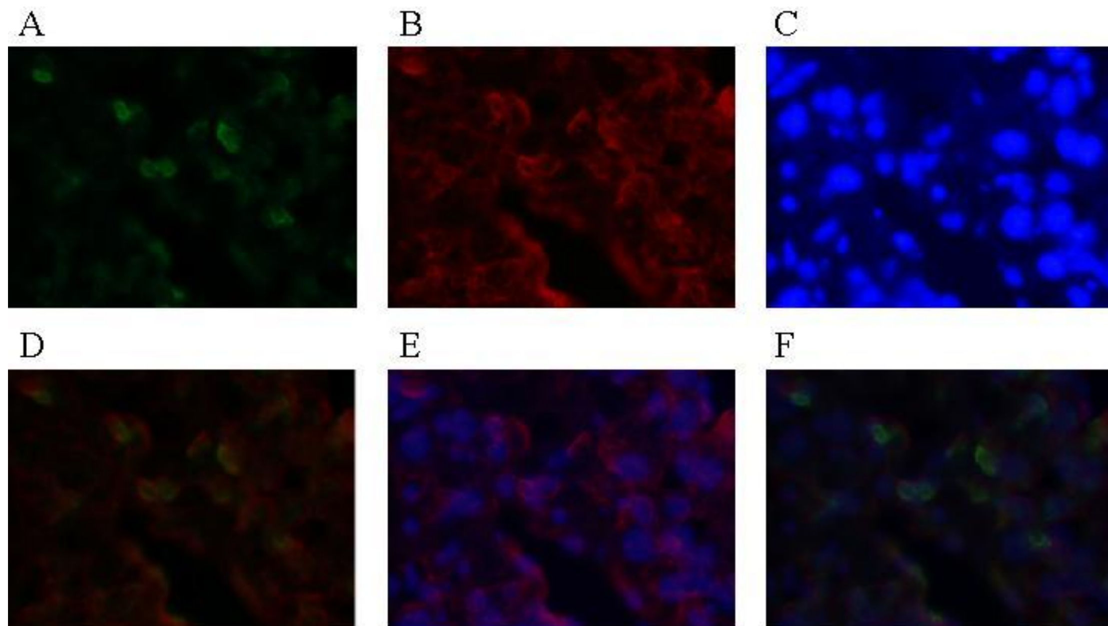

Fig. 15. IF stain of liver tissue from a recipient mouse (HM3-4).
Green fluorescence through anti-human mitochondria immune reaction (A).
Red fluorescence through anti-mouse albumin immune reaction (B). DAPI (C).
A merged with B (D). B merged with C (E). A, B and C merged together (F).

叁、心得

IUT in mice is a feasible model for researches of human AFS cells. Both fresh frozen human AFS cells and cultured human AFS cells were proved to engraft in fetal mice but fresh frozen human AFS cells are more pluripotent than cultured human AFS cells after transplantation. Certain physiological potentials like haematopoiesis may decline or even vanish while expanding human AFS cells *ex vivo* so that the cultured human AFS cells express only the characteristics of MSCs but not HSCs.

Further studies on larger numbers are required to confirm these initial findings. The larger quantity of transplanted human AFS cells is necessary for illuminating the correlation between dosage and engraftment rate. The sequential postmortem and evaluation of more organs will bring more information about human AFS cells. This experimental model may serve as a platform where the prerequisite for fetal therapies by autologous human AFS cells may move on.

肆、建議事項

The final objective of our researches is to apply AFS cells in fetal therapies, so more understanding of their i*n vivo* potentials in differentiation is definitely necessary. Although multilineage engraftments were demonstrated in this study, only 8 organs for evaluation is far from perfect to extrapolate the overall potentials of human AFS cells. More organs will be collected and examined in future experiments, especially the organs in which congenital diseases are involved. For example, evaluate the pancreas to see if there is a role for human AFS cells to treat congenital DM, evaluate bone for congenital skeletal disease, and evaluate brain or spinal cord for congenital CNS disease. It is imperative to dissect all the organs of therapeutic interests during postmortem at different ages of the recipient mice in the following studies.

Although this IUT model has been proved to be feasible and the transplanted human AFS cells were confirmed to engraft in fetal mice, it is still not clear that whether the cells of these engraftments have differentiated into the same lineage as the organ where they engraft, for example, have they become hepatocytes in mouse liver? Because the aim of stem cell therapy is to repair the damaged organs or to replace the impaired function, there is no point to perform IUT of human AFS cells if they just dwell there but not exert the proper function specific for the organ in which they engraft. According to the previously published article (Lee et al., 2004), it is important to extract RNA from the recipient mouse after IUT for RT-PCR to detect the expression of organ-associated genes specific for human, for example, human αFP gene and human albumin gene for liver. In immunofluorescence stain, besides the antibody to prove the existence of the transplanted human cells, the antibody specific to the function of respective lineage is required, for example, anti-human albumin antibody to prove the function of human hepatocytes.