＜別紙3＞

研究実績報告書

1. 招へいされた外国人研究者

所属・職名（和文）：肝臓疾患研究所(上海)・主任研究員

（英文）：Research Institute for Liver Diseases (Shanghai) Co. LTD,
Study Director

氏　　名（和文）：方 思詩

（英文）：Sishi Fang

2. 招へい申請者

所属・職名：札幌医科大学医学部附属がん研究所分子病理病態学部門・教授

氏　　名：三高 俊広

3. 受入研究者

所属・職名：札幌医科大学医学部附属がん研究所分子病理病態学部門・教授

氏　　名：三高 俊広

4. 招へい期間：平成17年12月4日～平成17年12月18日(15日間)

5. 研究課題：ヒト肝臓由来小型肝細胞の分離培養方法の確立

6. 研究活動の概要

12月 4日(日)：成田空港着、乗り換え後千歳空港に到着、札幌に移動後ホテルに入る。

12月 5日(月)：札幌医科大学記念ホール会議室において肝臓疾患研究所(上海)、札幌医科大学附属がん研究所分子病理病態学部門、第一化学薬品株式会社間の共同研究について会議を行う。

12月 6日(火)：札幌医科大学におけるラット小型肝細胞の調整方法の見学およびラット凍結小型肝細胞の培養方法の実習

12月 7日(水)：肝臓疾患研究所において調製されたヒト凍結肝細胞の培養および実験の準備（チトクローム P450 活性誘導実験）

12月 8日(木)：培養細胞の維持、実験準備

12月 9日(金)：実験、ディスカッション

12月10日(土)：実験、ディスカッション

12月11日(日)：サンプルの回収

12月12日(月)：2回目の実験のために細胞を準備する

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12月13日(火):実験、ディスカッション

12月14日(水):サンプルの回収、まとめ

12月15日(木):ホテルをチェックアウトし、千歳空港に移動する。羽田空港に着き、そのままホテルへ移動。

12月16日(金):第一化学薬品株式会社本社会議室にて二宮真一部長および関係者と共同研究打ち合わせ

12月17日(土):休日

12月18日(日):成田空港より出国

7. 研究課題の成果

本報告書の受け入れ研究室である札幌医科大学医学部附属がん研究所分子病理病態学部門ではラット小型肝細胞の研究を行ってきた。小型肝細胞は成体肝臓に存在する肝細胞中に数パーセントの割合で存在し、肝細胞としての機能を保持し、かつ増殖力の高い肝前駆細胞である。小型肝細胞は、星細胞などの肝非実質細胞との共培養により分泌された細胞外基質の作用により成熟細胞へと分化する。成熟化した小型肝細胞は、チトクローム P450 等、主要な薬物代謝酵素の活性を一ヶ月以上培養した状態でも発現誘導することができ、従来ラット初代培養肝細胞では反応しないとされていたリファンピシンによる CYP3A4 の誘導も起こる。また成熟した小型肝細胞間には毛細胆管が形成され、できた毛細胆管面には MDR や MRP2 などのトランスポータータンパク質が局在し、細胞内で代謝された産物を毛細胆管中に分泌している。更に小型肝細胞は凍結保存が可能であり、6ヶ月以上凍結保存した細胞を増殖可能である。

国内及び国外においても薬物動態の研究に肝細胞を用い、薬物代謝酵素活性の測定を行っている研究室は多い。しかし、初代培養肝細胞やトランスポーター遺伝子を導入した細胞株を用いて研究を行っている。初代培養肝細胞は培養経過と共に高度な分化機能と考えられているアミノ酸代謝酵素活性や薬物代謝酵素活性、特にチトクローム P450 の活性が急速に低下することが知られている。また肝細胞膜の極性も失われるため毛細胆管が形成されないばかりか、細胞内での物質の代謝機構、ソーティング機構も正常細胞とは異なっていると考えられる。また、遺伝子導入すると細胞内のホメオスターシスが正常とはかなり異なることが予想され、生体内で起こりうる機序を推定するにはまだ十分な方法とは言い難い。我々は、小型肝細胞を用いることによってこれらの問題を解決できると考えている。小型肝細胞はヒトにも存在することが本部門の研究結果により明らかとなっているが、現状では日本ではヒトの肝臓を手に入れるのは難しく、癌の肝転移症例において外科手術時に切除した肝組織の一部分を使って実験を行っているのが現状である。薬物動態に関してもラットなど齧歯類とヒトとでは必ずしも同じではないことが知られているため、恒常的にヒトの肝臓が入手できると今後の研究も飛躍的に亢進すると思われる。

中国上海の Research Institute for Liver Diseases (Shanghai、RILD 社)は臓器提供カードの記載を基に心臓死直後のヒト肝臓の提供を受け、肝細胞の調整を行っている。従来調整していたのは凍結保存した成熟肝細胞であったが、上述のように成熟肝細胞では融解後すぐに使用することが必須となる。そこで、第一化学薬品株式会社及び分子病理病態学部門との共同研究により増殖し、且つ長期培養可能な小型肝細胞

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をヒト肝臓より分離培養する方法を確立することが提案され、共同研究を始める運びとなり、今回RILD社より実際に肝細胞を調製し研究しているFang先生を招へいし、RILD社におけるヒト凍結肝細胞の融解及び播種方法を教授してもらうばかりではなく、ヒト小型肝細胞の分離培養する方法の確立するために共同で実験を行った。

札幌医大のプロトコールと中国における細胞の解凍方法、培養方法にわずかな違いがあり、マニュアル通りに行っても当教室では上手くいかなかったことが、Fang先生がデモンストレーションした方法と同様に行うと上手くいくことがわかった。教授前、当教室で融解及び播種を行った際には細胞がほとんど死んでしまったのが、教授後は8割近い細胞が培養皿に生着していることが確認された。実際に実験を行っている研究者を呼ぶことによってマニュアルには表現されていないコツを教授されたことと実験方法の差異の確認ができたことは今後の共同研究を進める上で重要なことと考えられる。しかしながら、まだ完全に再現されてはいないので、今後は培養液の組成も検討しながらヒト小型肝細胞の長期培養を行い、小型肝細胞のコロニーが多数出現する培養条件の検討を行うことが話しあわれた。今回の招へい期間中にFang先生がラット小型肝細胞の調整法を見学したので、ヒト小型肝細胞の調整法と比較し、ヒト小型肝細胞の調整法の再検討も行う必要があるとの共通認識も得られた。

RILD社では凍結肝細胞及び小型肝細胞の機能評価を薬物代謝酵素活性で行うこととした。今回の招へい期間中に行った実験の結果得られたサンプルを解析し、RILD社において行われる実験の結果と比較検討することにより、今後両研究施設において出されるデータを共通データとして用いることが可能になる。本実験の解析結果は第一化学薬品株式会社薬物動態研究所にて解析中である。

今後は、この成果をふまえて共同研究を進めていく考えである。

8. 外国人研究者のレポートを次に示す。

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外国人研究者によるレポート

Project Title: Preparation and application of in vitro human liver systems into biopharmaceutical research

Reporter: Sishi Fang, M.D, Study Director, Research Institute for Liver Diseases (Shanghai) Co. Ltd., Shanghai, China (RILD)

Project Period: From Dec. 4, 2005 to Dec. 18, 2005

Project Activities:

Dec. 4, 2005: Fly from Shanghai to Narita and then to Sapporo

Dec. 5: Sapporo Medical University

Presentation of my own research in RILD to Prof. Mitaka's laboratory member and discussion with them.

Dec. 6: Sapporo Medical University

Discussion about the detail of my experiments in Sapporo with Prof. Mitaka and Dr. Kon.

- Preparing and plating rat small hepatocytes into collagen coated dishes.
- Preparing human small-sized and mature hepatocytes into collagen coated dishes.
- Thawing cryopreserved rat small hepatocytes and replating in collagen coated culture plates.
- Preparation of solutions by the RILD Standard Operation Procedure.

Dec. 7: Sapporo Medical University

- Preparing and plating rat small hepatocytes into collagen coated dishes.
- Observing cell attachment and cell density of cryopreserved rat small hepatocyte colonies.
- Thawing cryopreserved human small-sized hepatocytes (RILD Lot

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No.HH-05-002s; See Table 1 and Figure 1) and replating them into collagen coated culture plate.

- Preparation of the stock solution for CYP-induction study.

Dec. 8: Sapporo Medical University

- Morphological analysis of the colony attachment.
- Plating Matrigel on rat small hepatocyte colonies and replacing incubation medium.
- Dosing human small-sized hepatocytes with selective inducers of CYP3A4 and CYP1A2 (Three-day induction procedure, Day 1).

Dec. 9: Sapporo Medical Uniersity

- Morphological analysis of the cell attachment and replacing incubation medium.
- Dosing human small-sized hepatocytes with selective inducers of CYP3A4 and CYP1A2 (Three-day induction procedure, Day 2).

Dec. 10: 2005, Sapporo Medical University

- Morphological analysis of the cell attachment and replacing incubation medium.
- Dosing human small-sized hepatocytes with selective inducers of CYP3A4 and CYP1A2 (Three-day induction procedure, Day 3).

Dec. 11: 2005, Sapporo Medical University

- Phenotyping and characterization of CYP1A2 and 3A4 by using selective substrates for human small hepatocytes. (See Table 4 and Table 5)
- Morphological analysis of the cell attachment and replacing incubation medium.

Dec. 12: 2005, Sapporo Medical University

Thawing the cryopreserved human small-sized hepatocytes from Chinese donors, and

- replating them in collagen coated culture plate.
- Dosing human small-sized epatocytes with selective inducer of CYP3A4

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and CYP1A2 (Four-day induction procedure, Day 1).

- Comparison between Prof. Mitaka's and RILD methods of the induction.

Dec. 13: 2005, Sapporo Medical University

- Dosing human small-sized hepatocytes with selective inducer of CYP3A4 and CYP1A2 (Four-day induction procedure, Day 2).
- Comparison between Prof. Mitaka's and RILD methods of the induction.

Dec. 14: 2005, Sapporo Medical University

Summarize the data and discuss with Prof. Mitaka.

- Dosing human small-sized hepatocytes with selective inducer of CYP3A4 and CYP1A2 (Four-day induction procedure, Day 3).
- Comparison between Prof. Mitaka's and RILD methods of the induction.

Dec. 15: Fly to Tokyo

Dec. 16: Meeting with collaborators of Daiichi Pure Chemical Co.

Dec. 17-18: Holiday and preparation for leaving.

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Results of Collaborative Research

Figure 1. Comparison of previous culture of cryopreserved hepatocytes HH-05-002 (regular-sized cell) and HH-05-002s (small-sized cell) after5 day incubation by RILD

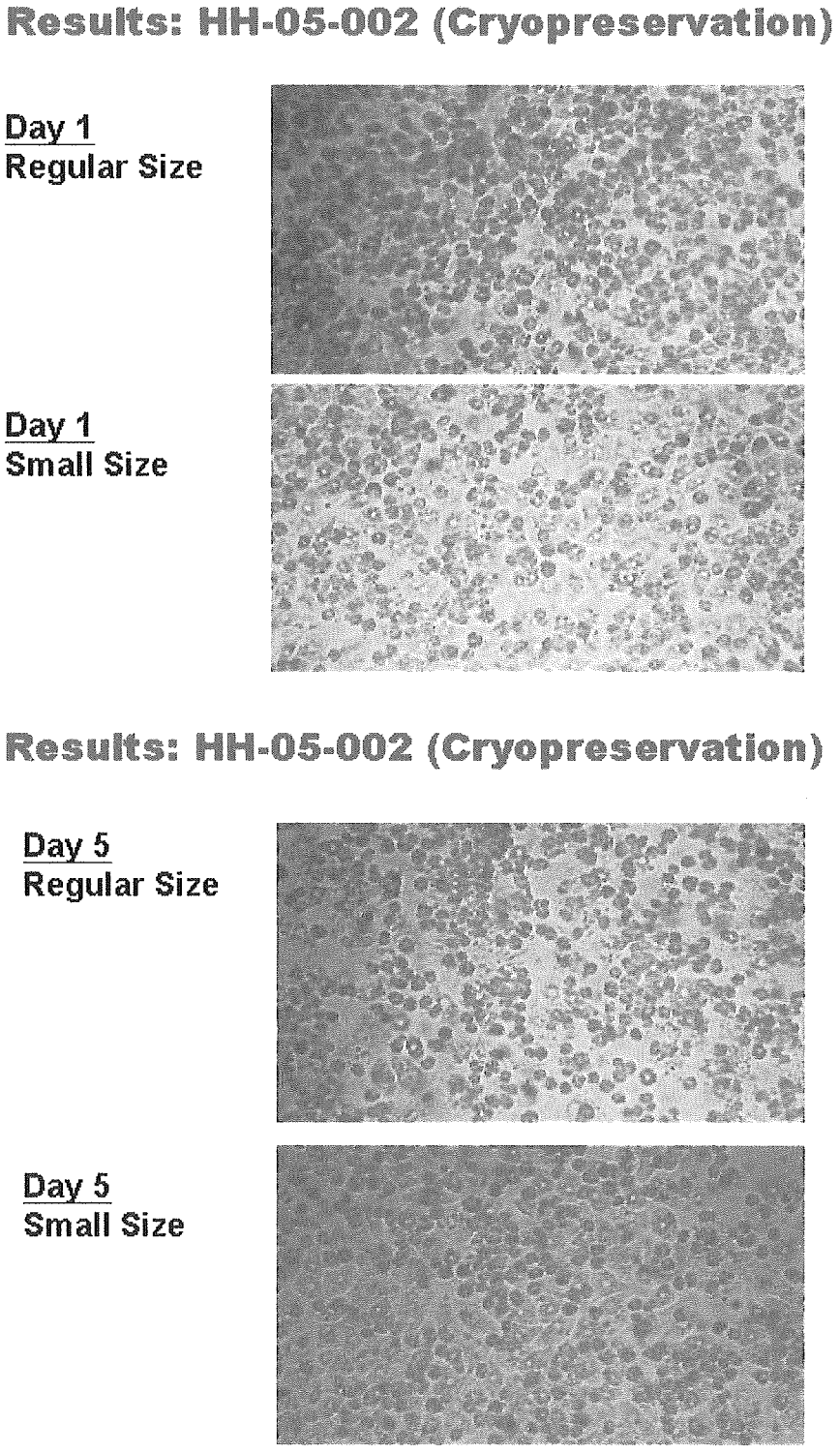

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Table 1. In vitro testing systems in RILD

In Vitro Testing Systems

In vitro systems	Description	Size
Cryopreserved hepatocytes	Human, SD rat, Beagle dog, Cynomolgus monkey	5 millions / vial
Replatable Cryo- hepatocytes	Human (Regular & Small Size)	5 millions
Plated cultured hepatocytes	Human, SD rat, Beagle dog, Cynomolgus monkey	6/12/24/96 well plate
Hepatocytes in suspension	Human, SD rat, Beagle dog, Cynomolgus monkey	10 millions / tube
Liver microsomes	Human, SD rat, Beagle dog, Cynomolgus monkey	20 mg protein/mL
Liver / kidney precise cut slices *Cultured & Cryopreserved*	Human, SD rat, Beagle dog, Cynomolgus monkey	300 uM x 10 x 10 mm

Table 2. Donor information an results of serology testing from RILD

Donor Information

▪Donor Number	▪HH-05-002
▪Gender	▪Male
▪Age	▪32
▪Race	▪Mongolian
▪Cause of Death	▪Trauma
▪Smoker	▪N/A
▪Medical History	▪N/A

Results of Serology Testing

▪HIV 1	▪HIV 2	▪HTLV	▪HTLV	▪HBV	▪HCV	▪CMV	▪RPR
▪-	▪-	▪N/A	▪N/A	▪-	▪-	▪N/A	▪-

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Table 3. Previous result comparison of cryopreserved hepatocytes by RILD

Day 1 in suspension incubation

▪Lot No.	▪Viability (%)	▪Formation of Metabolites	
		▪1A2	▪3A4
		▪Acetaminophen (pmol/10^6/min)	▪6-β-OH Testosterone (pmol/10^6/min)
▪HH-05-002 small	▪78%	▪36.16±0.93	▪51.33±2.26
▪HH-05-002 normal	▪74%	▪12.01±0.98	▪11.83±9.63

Table 4. Previous characterization comparison of cryopreserved hepatocyte HH-05-002 and HH-05-002s on CYP1A2 after 3-day induction by RILD

▪Activity of CYP1A2: Formation of Acetaminophen (pmol/10^6/min)		
▪Cell Type	▪Small	▪Regular
▪Viability	▪78%	▪74%
▪NC	▪6.45±2.51	▪BLQ
▪Omeprazole 25μM	▪13.44±1.46	▪BLQ
▪Omeprazole 50μM	▪15.49±1.84	▪BLQ
▪Omeprazole 100μM	▪12.90±1.04	▪8.17±0.46

Table 5. Previous characterization comparison of cryopreserved hepatocyte HH-05-002 and HH-05-002s on CYP34 after 3-day induction by RILD

▪Activity of CYP3A4: Formation of 6-β-hydroxytestosterone (pmol/10^6/min)		
▪Cell Type	▪Small	▪Regular
▪Viability	▪78%	▪74%
▪NC	▪1.65±2.86	▪BLQ
▪Rifampcin 12.5μM	▪27.93±1.46	▪9.68±1.57
▪Rifampcin 25μM	▪42.33±4.96	▪22.1±1.96
▪Rifampcin 50μM	▪49.19±2.36	▪46.7±4.96

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Summary of collaborating research in Sapporo

1. This project functioned significantly in exchanging research experiences in terms of both scientifically and ethically between Chinese Scientists and Japanese Scientists.

2. This project promoted the experience exchanges not only in the level of Directors such as Prof. Mitaka and Prof. Hu, but also in level of research scientists such as Dr. Fang in Research Institute for Liver Diseases (Shanghai) Co, Ltd, China and Drs. Kon, Ooe, and Chen in Sapporo Medical University, Japan, especially in terms of technical details as experimental procedures, reagents, equipments, and facility.

3. This project funded a solid base for future cooperation in area of applying in vitro liver system into medical and pharmaceutical research between Research Institute for Liver Diseases (Shanghai) Co, Ltd, China and Prof. Mitaka's Laboratory at Sapporo Medical University, Sapporo, Japan.

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平成１７年度厚生労働省科学研究費補助金第一回研究会議

萌芽的先端医療技術推進研究事業：トキシコゲノミクス分野
研究課題名：肝ステム細胞を用いた毒性発現の評価解析方法の確立
研究代表者：札幌医科大学がん研究所分子病理病態学部門
教授　三高　俊広

日時：平成１７年１２月５日（月曜日）
場所：札幌医科大学記念ホール会議室Ｂ

参加者：１１名

Research Institute for Liver Diseases (RILD 社)
Dr. Zhuohan HU, Ph.D. (CEO/CSO)
Dr. Shishi Fang, M.D. (Study Director)

第一化学薬品株式会社薬物動態研究所
二宮真一（研究開発部長・研究協力者）

がん研究所分子病理病態学部門
三高俊広（教授・主任研究者）
今　純子（助手・研究協力者）
陳　其潔（流動研究員・研究協力者）
大栄秀和（訪問研究員・研究協力者）
大島秀紀（研究生・研究協力者）
佐々木寿誉（研究生・研究協力者）
高松みのり（訪問研究員・研究協力者）
市戸義久（大学院生・研究協力者）

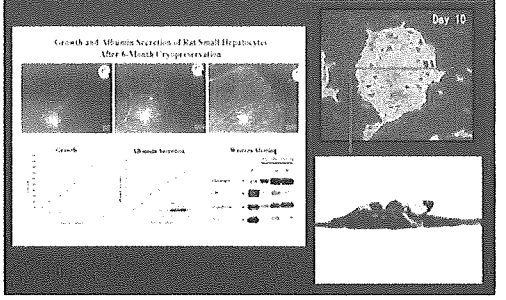
会議資料：（次ページより）

1. **大栄秀和**
2. **今　純子**
3. **Zhuohan Hu**

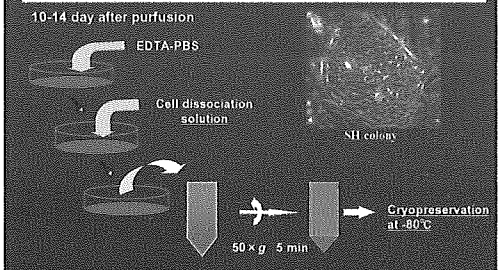
1. 大栄 秀和
（札幌医科大学附属がん研究所分子病理病態学部門）

Cytochrome P450 Expressions of Cultured Rat Small Hepatocytes After Long-Term Cryopreservation

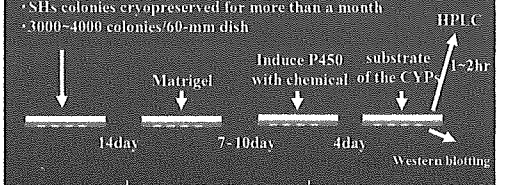
Culture of cryopreserved small hepatocytes



Isolation and cryopreservation of SH colonies

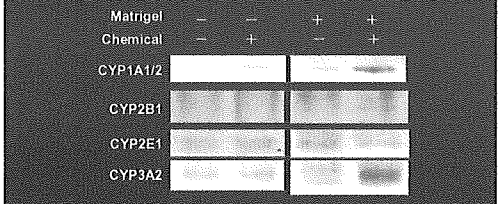


Measurement of CYPs expression and activity

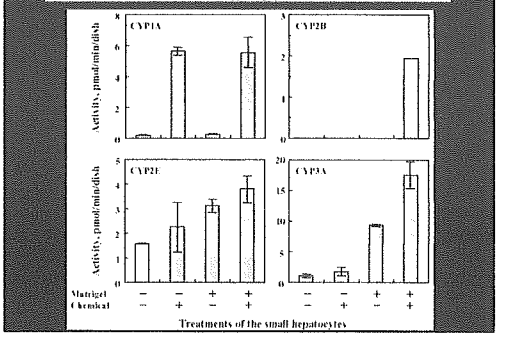


	Chemical	Substrate
CYP1A	5 μM 3-methylcholanthrene	3 μM ethoxyresorufin
CYP2B	2mM phenobarbital	125 μM testosterone
CYP2E	100mM ethanol	300 μM chlorzoxazone
CYP3A	2 μM pregnenolone-16α-carbonitrile	125 μM testosterone

Immunoblots for CYPs



CYPs activity in cryopreserved small hepatocytes



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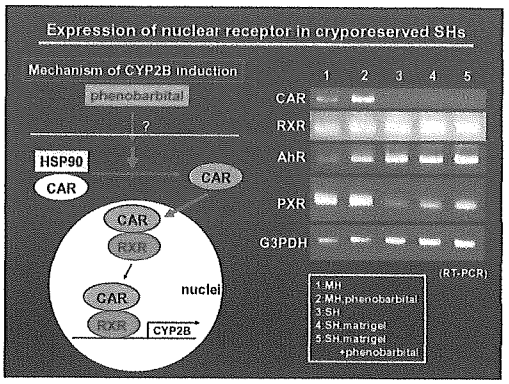


Results

- Cryopreserved SHs constitutively expressed CYP1A1/2, CYP2E1, and CYP3A2 proteins, whereas CYP2B1 was not detected.
- CYP1A, 2E, 3A activities were also detected.
- CYP1A and CYP3A were induced by chemicals in the SHs.
- CYP2B expression and activity were not detected.
- Defects of CYP2B may be caused by the suppression of CAR.

2　今　純子
（札幌医科大学附属がん研究所分子病理病態学部門）

小型肝細胞におけるCD44の発現とその機能解析

Experiments in Department of Pathophysiology

Rat SH

1. Analysis of specific markers in SHs (Sasaki, Chen, Kon,).
2. Differentiation of rat progenitor cells using injury model (Oshima, Sasaki, Kon).
3. Analysis of drug metabolization using SHs (Ono, Oshima).
4. Establishment of the cholestasis model using SHs (Oshima).

Human SHs

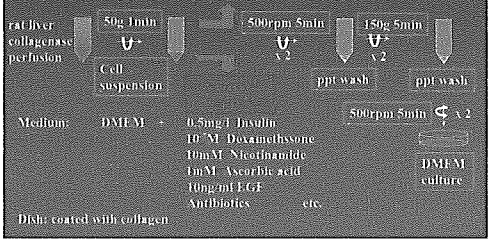
1. Establishment of SH preparation and culture (Sasaki).
2. Identification of specific markers in SHs (Sasaki,).

Scaffolds

1. Films, gels and sponges consist of ocean collagen (Takamatsu).
2. Hydroxyapatite coated titanium (Ichinohe).

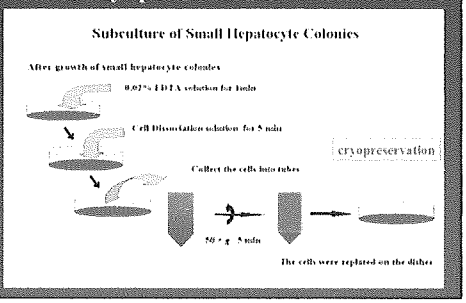
Methods: Preparation of SH

Rat F344/NSlc, SD
Methods:



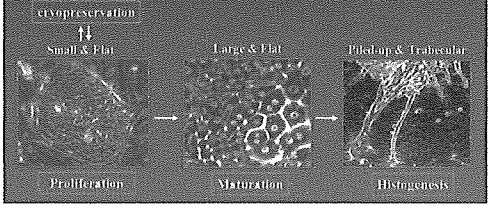
Dish: coated with collagen

Methods: Subculture and cryopreservation of SH

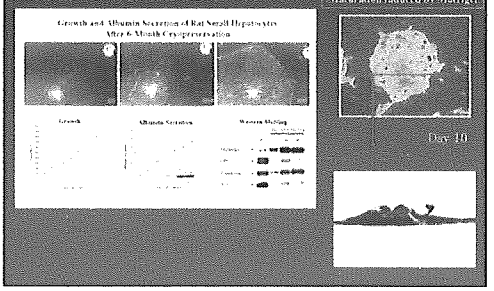


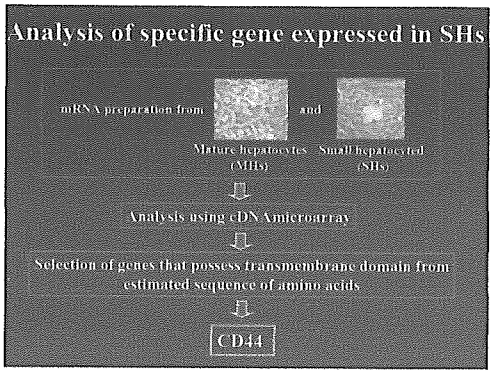
Characterization of SH 1

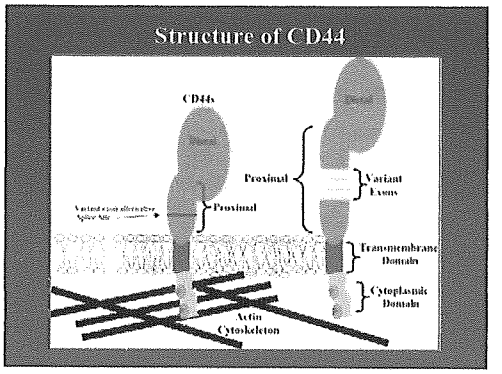
1. Clonal expansion in the culture when isolated from adult rat liver
2. Maturation occurs in the mixed culture with non-parenchymal cells
3. Piled-up and make the trabecular structure
4. Can be cryopreserved
5. Exists in human liver

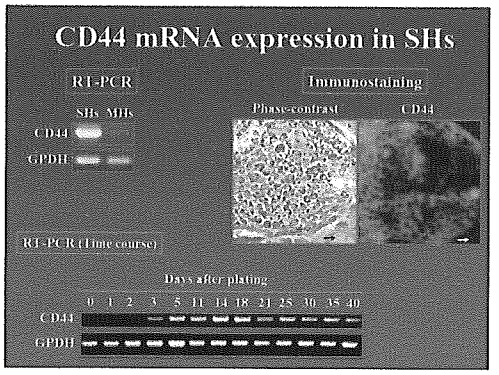


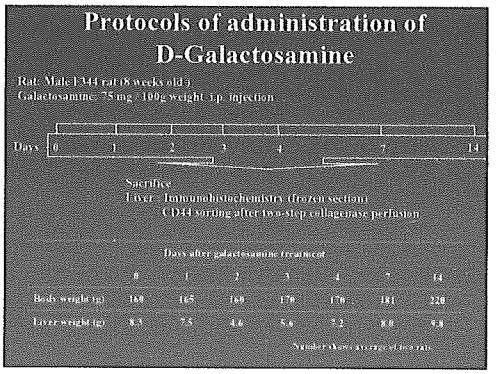
Characterization of SH 2

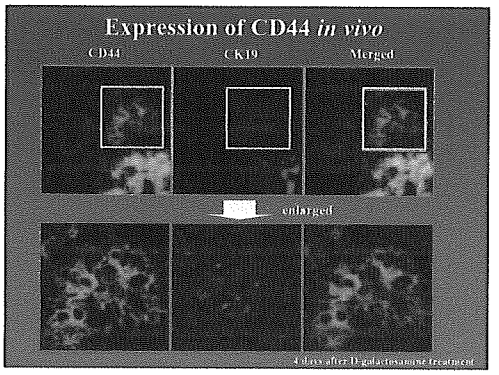


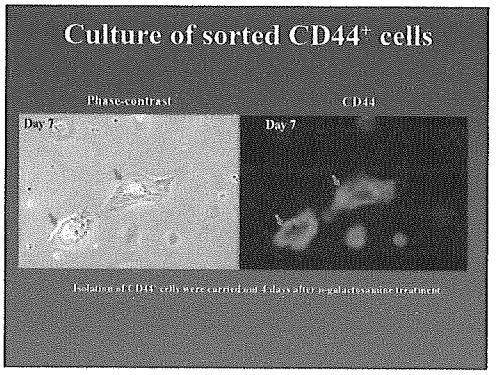

Analysis of specific gene expressed in SHs
mRNA preparation from
and
Mature hepatocytes (MHs)
Small hepatocyte (SHs)
Analysis using cDNA microarray
Selection of genes that possess transmembrane domain from estimated sequence of amino acids
CD44

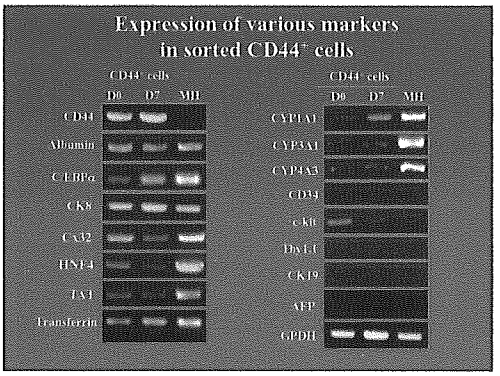

Structure of CD44
CD44s
Proximal
Variant Exons
Proximal
Transmembrane Domain
Cytoplasmic Domain
Actin Cytoskeleton


CD44 mRNA expression in SHs
RT-PCR
Immunostaining
SHs MHs
CD44
GPDH
Phase-contrast
CD44
RT-PCR (time course)
Days after plating
0 1 2 3 5 11 14 18 21 25 30 35 40
CD44
GPDH


Protocols of administration of D-Galactosamine
Rat: Male Lewis rat (8 weeks old)
Galactosamine: 75 mg / 100g weight i.p. injection
Days
0 1 2 3 4 7 14
Sacrifice
Liver: Immunohistochemistry (frozen section)
CD44 sorting after two-step collagenase perfusion
Days after galactosamine treatment
0 1 2 3 4 7 14
Body weight (g) 160 165 160 170 170 181 220
Liver weight (g) 8.3 7.5 4.6 5.6 7.2 8.0 9.8


Expression of CD44 in vivo
CD44
CK19
Merged
enlarged
4 days after D-galactosamine treatment


Culture of sorted CD44+ cells
Phase-contrast
CD44
Day 7
Day 7



Summary

1. Expression of CD44 was specific in SHs

2. CD44$^+$ cells appeared in periportal region *in vivo*

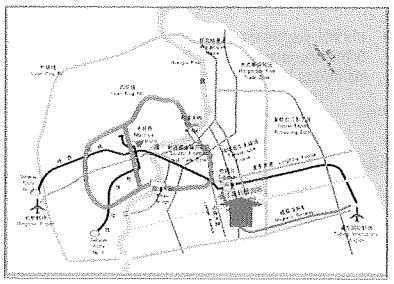
3. Sorted CD44$^+$ cells formed colonies like SHs and possessed hepatic markers

Research & Development of Innovative Therapeutics by using in vitro systems

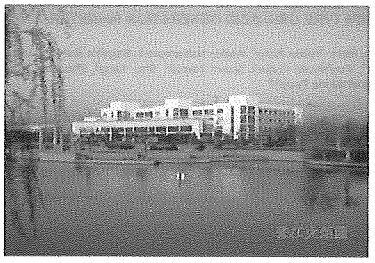
Zhuohan Hu[1,2], Ph.D. and Shisi Fang[1], M.D.

1. Research Institute for Liver Diseases (Shanghai)
2. School of Pharmacy, Shanghai Fudan University
Shanghai, China

Location: Shanghai Zhangjiang HighTech Park



Facility: National Incubator for Biotech Institutes



Content

1. **In vitro systems: preparation, validation, & shipment**
2. **In vitro drug metabolism and interaction: case studies**
3. **Ethical and Legal requirements and regulations on using human tissues in China**

Our Mission

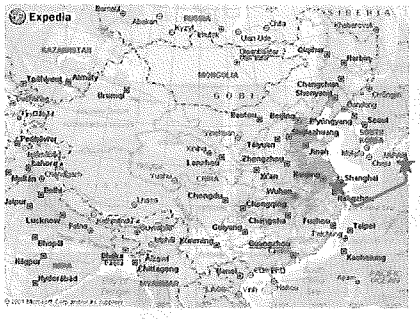
In Vitro Liver / Kidney Systems
(Shanghai Project No. 20040108)

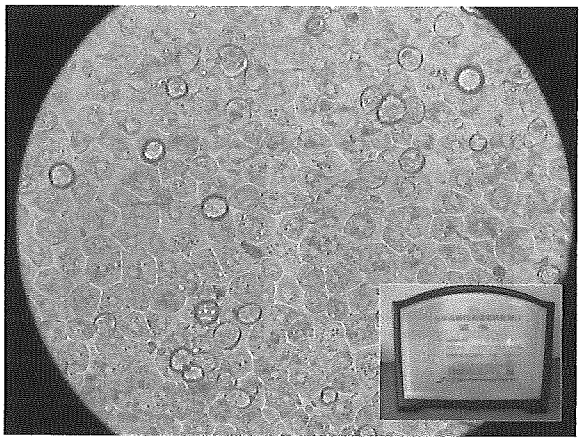
- In vitro evaluation for drug metabolism & interaction — State Project No. 03C226213100901
- Artificial Liver Dialysis - Supporting Liver transplanting — Shanghai Project No. PKJ2003-044
- In vitro screening for antiviral therapeutics — State Project No. 2004BA719A09-04a

In Vitro Testing Systems

In vitro systems	Description	Size
Cryopreserved hepatocytes	Human, SD rat, Beagle dog, Cynomolgus monkey	5 millions / vial
Replatable Cryo- hepatocytes	Human (Regular & Small Size)	5 millions
Plated cultured hepatocytes	Human, SD rat, Beagle dog, Cynomolgus monkey	6/12/24/96 well plate
Hepatocytes in suspension	Human, SD rat, Beagle dog, Cynomolgus monkey	10 millions / tube
Liver microsomes	Human, SD rat, Beagle dog, Cynomolgus monkey	20 mg protein/mL
Liver / kidney precise cut slices *Cultured & Cryopreserved*	Human, SD rat, Beagle dog, Cynomolgus monkey	300 uM x 10 x 10 mm

Location





Fresh Hepatocytes in suspension or cultured in plates

- 2 x 10^7 per 50 mL in RILD suspension media
- Shipping temperature: 2-8 °C
- Shipping time: 60 hours

Day 1 9:00 am: YAMATO, Shanghai Pudong International Airport
13:00pm: Custom clearance
16:10pm: on board of JL610
19:30pm: Narita International Airport, Tokyo
Day 2 9:30 am: Custom clearance
13:30pm: Arrival at DPC Research Institute of ADME/T (东岩村)
17:00pm: 北海道大
18:00pm: 金沢大

RILD 肾脏肝病研究	TILTE: Preparation and Characterization of small size hepatocytes
Project Note (4)	Protocol No: RD-2005-JP-004-PT1 Version No: 1

OBJECTIVE
To prepare and characterize small size hepatocytes.

SYSTEM:
Human

PROCEDURE
1. Preparation of hepatocytes (RILD SOP-P-002)
2. Isolation of small size hepatocytes
3. Cryopreservation and thawing (RILD SOP-P-009)
4. Plating hepatocytes(RILD –SOP-A-002)
5. Incubating and inducing hepatocytes (RILD-SOP-P-013)

CARRIER-MEDIATED HEPATIC UPTAKE OF A NOVEL NON-RENAL EXCRETION TYPE URIC ACID GENERATION INHIBITOR, Y-700

Yoshimichi Sai1, Yukio Kato[1], Keiko Nakamura1, Sayaka Kato[1], Tomohiro Nishimura[1], Yoshiyuki Kubo1, Ikumi Tamai[2], Shu Yang[3], Zhuohan Hu[3], Ichimaro Yamada[4] and Akira Tsuji[1,5]

1. Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan.
2. Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
3. Research Institute for Liver Diseases (Shanghai), Shanghai 328 Bibo Road, C101-113, Shanghai 201203, China
4. Pharmacokinetics Laboratory, Pharmaceuticals Research Division, Mitsubishi Pharma Corporation, 1-1-1 Kazusakamatari, Kisarazu, Chiba 292-0812, Japan
5. To whom correspondence should be addressed. (e-mail: tsuji@kenroku.kanazawa-u.ac.jp)

RILD 肾脏肝病研究	TILTE: Preparation and Characterization of small size hepatocytes
Project Note (4)	Protocol No: RD-2005-004-PT1 Version No: 1

Results: HH-05-001

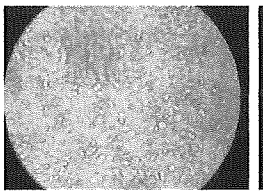


Figure1-a. Day0-Normal cell-25x

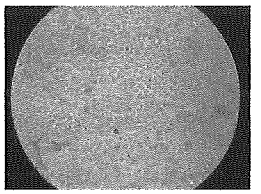


Figure1-b. Day0-Small cell-25x

Identification of a Novel Enhancer of the Human 1A1 Gene

Norhito Shibahara, Kaoru Nagata, Shunsuke Iwano, Tetsuya Saito, Kazuma Kiyotani, Goro Honda, Kazuko Nakagawa, Zhuohan Hu, Noriaki Shimada and Tetsuya Kamataki

RILD 肾脏肝病研究	TILTE: Preparation and Characterization of small size hepatocytes
Project Note (4)	Protocol No: RD-2005-JP-004-PT1 Version No: 1

Results: HH-05-001

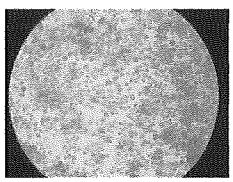


Figure3-a. Day5-Large cell-25x

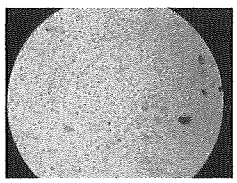
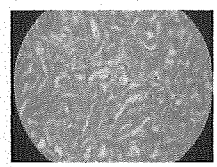
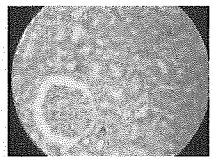


Figure5-b. Day8-Small cell-25x

Cryopreserved Human Kidney Precise Cut Slices



Before Cryopreservation



After thawing from -150°C

FUNCTIONAL ANALYSIS OF TRANSPORTERS USING HUMAN KIDNEY SLICES

Yasuhisa Adachi[1], Sishi Fang[2], Zhuohan Hu[2], Shinichi Ninomiya[1] and Tetsuji Sudo[1]

1 ADME/Tox Research Institute, Daiichi Pure Chemicals Co., Ltd. 2117 Miyamatsu, Tokai-mura, Ibaraki-ken 319-1162, Japan
2 Research Institute Liver Disease (Shanghai), 328 Bi Bo Road C109, Shanghai, P.R. China, 201203

Results

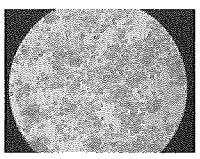


Figure4-a. Day8-Large cell-25x

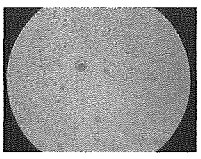
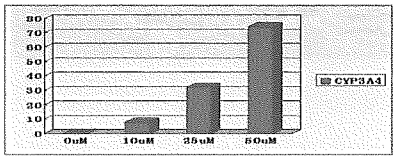


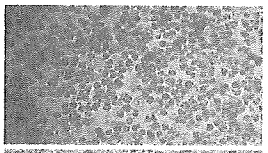
Figure8-b. Day4-Small cell-25x



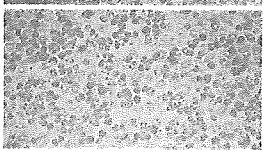
<別紙４>

Results: HH-05-002 (Cryopreservation)

Day 1
Regular Size



Day 1
Small Size



Three Day Induction Study: CYP3A4

Activity of CYP3A4: Formation of 6-β -hydroxytestosterone (pmol/10⁶/min)		
Cell Type	Small	Regular
Viability	78%	74%
NC	1.65 ± 2.86	BLQ
Rifampicin 12.5μM	27.93 ± 1.46	9.68 ± 1.57
Rifampicin 25μM	42.33 ± 4.96	22.1 ± 1.96
Rifampicin 50μM	49.19 ± 2.36	46.7 ± 4.96

Results: HH-05-002

Donor Information

Donor Number	HH-05-002
Gender	Male
Age	32
Race	Mongolian
Cause of Death	Trauma
Smoker	N/A
Medical History	N/A

Results of Serology Testing

HIV 1	HIV 2	HTLV 1	HTLV 2	HBV	HCV	CMV	RPR
-	-	N/A	N/A	-	-	N/A	-

Three Day Induction Study: CYP1A2

Activity of CYP1A2: Formation of Acetaminophen (pmol/10⁶/min)		
Cell Type	Small	Regular
Viability	78%	74%
NC	6.45 ± 2.51	BLQ
Omeprazole 25μM	13.44 ± 1.46	BLQ
Omeprazole 50μM	15.49 ± 1.84	BLQ
Omeprazole 100μM	12.90 ± 1.04	8.17 ± 0.46

Results: HH-05-002 (Cryopreservation)

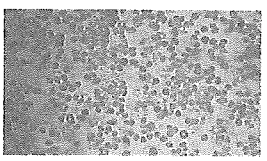
Day 1 in suspension incubation

Lot No.	Viability (%)	Formation of Metabolites	
		1A2	3A4
		Acetaminophen (pmol/10⁶/min)	6-β -OH Testosterone (pmol/10⁶/min)
HH-05-002 small	78%	36.16 ± 0.93	51.33 ± 2.36
HH-05-002 normal	74%	12.01 ± 0.93	11.03 ± 0.63

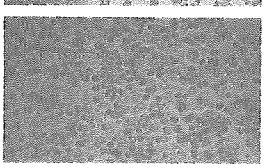
HH-05-002: Arrival at 20:05 pm

Results: HH-05-002 (Cryopreservation)

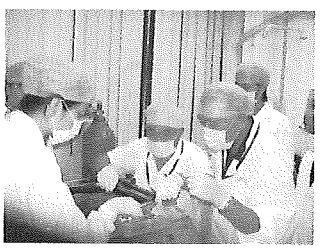
Day 5
Regular Size



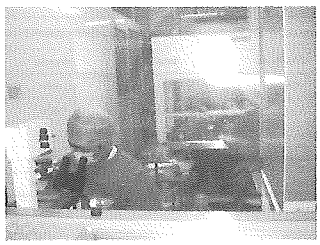
Day 5
Small Size



HH-05-002: Digestion at 20:35 pm



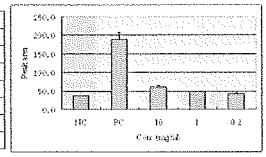
HH-05-002: Cell counting at 23:25 pm



ADMET-3

In vitro evaluation for inductive potential of test article on human P450 Isoforms
Test System: Human Primary Hepatocytes
Test Endpoints: Cytochrome P450s

CYP Isoforms	Enzyme Reactions
1A2	Phenacetin demethylation
2A6	Coumarin 7-hydroxylation
2C9	Tolbutamide 4-hydroxylation
2C19	S-mephenytoin 4-hydroxylation
2D6	Dextromethorphan O-demethylation
2E1	Chlorzoxazone 6-hydroxylation
3A4	Testosterone 6-β-hydroxylation

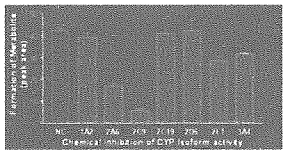


HH-05-002: Completion at 01:48 am

ADMET-5

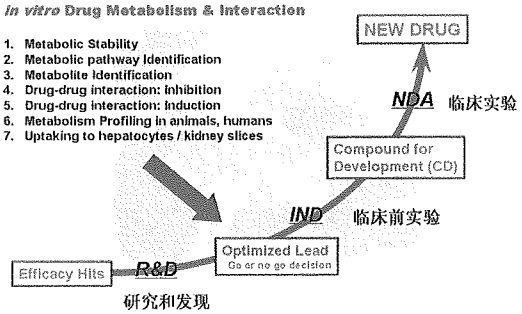
In vitro identification of metabolism pathway of test article
Test System: Human Cryopreserved Hepatocytes / or microsomes
Test Endpoints: Cytochrome P450s

CYP Isoforms	Enzyme Reactions	Selective Inhibitor
1A2	Phenacetin demethylation	Furafylline
2A6	Coumarin 7-hydroxylation	Tranylcypromine
2C9	Tolbutamide 4-hydroxylation	Sulfaphenazole
2C19	S-mephenytoin 4-hydroxylation	Omeprazole
2D6	Dextromethorphan O-demethylation	Quinidine
2E1	Chlorzoxazone 6-hydroxylation	4-methylpyrazole
3A4	Testosterone 6-β-hydroxylation	Ketoconazole

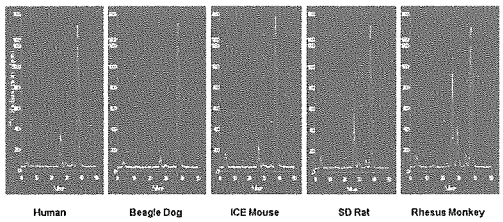


In vitro Drug Metabolism & Interaction

1. Metabolic Stability
2. Metabolic pathway Identification
3. Metabolite Identification
4. Drug-drug interaction: Inhibition
5. Drug-drug interaction: Induction
6. Metabolism Profiling in animals, humans
7. Uptaking to hepatocytes / kidney slices



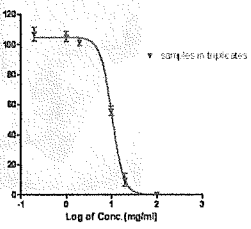
ADMET-7: In vitro evaluation of metabolism profiling of test article in animals and humans (Cryopreserved hepatocytes / Liver microsomes)



ADMET-1

In vitro evaluation for inhibitory potential of test article on human liver microsomal cytochrome P450 Isoforms
Test System: Human Liver Microsomes
Test Endpoints: Cytochrome P450s

CYP Isoforms	Enzyme Reactions
1A2	Phenacetin demethylation
2A6	Coumarin 7-hydroxylation
2C9	Tolbutamide 4-hydroxylation
2C19	S-mephenytoin 4-hydroxylation
2D6	Dextromethorphan O-demethylation
2E1	Chlorzoxazone 6-hydroxylation
3A4	Testosterone 6-β-hydroxylation



R & D program – Artificial liver dialysis